The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Innervation of Human Back Skin

By

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(Received for publication, September 12, 1953)

The histological study of skin innervation has recently been greatly promoted by Prof. Seto[1),2),3),4),5),6)] and his disciples, resulting in many a noteworthy achievement, but so far, the skin on the back has not been subjected to such a research. As far as I am aware, this part of the skin has been very little studied, if any, since olden times.

In view of such a situation, I took the materials of my study from the back skins of three human cadavers, with preference of the skin covering the area between the 5th and 8th thoracal spinal processes, and having fixed them for a long time in neutral formol, cut them into 40μ sagittal and horizontal frozen sections, and stained them with Seto's silver method. The large series of tissue preparations thus obtained I subjected to microscopic examination and succeeded in obtaining the observations described hereunder.

Individual Observations

According to Prof. Seto[1),2),3),5),6)] and his collaborators, the nerve elements, in particular, the terminal formations of sensory fibres in the hairless skin, e.g., on the palms and the soles, are widely different from those in the haired skin. In the former, such specific corpuscular terminations as Meissner's and Pacinian bodies are formed (Wada)[4)], while in the latter, the sensory nerve fibres end in most cases in connection with hairs, only an infinitesimally small portion reaching into the papillar layer of the corium to end there in unbranched or simple branched terminations, except at the external genitals (Hotta)[5)] and the papilla mammae and areola mammae (Suga)[6)], where genital nerve corpuscles and minor Pacinian bodies are found.

The poor development of the sensory nerve fibres in the papillar layer is histologically related to the poor formation of subepidermal papillae, as has been established beyond doubt by so many studies by Seto and his co-workers. So, whereas in the hairless skin and the skin covering the external genitals, the papilla mammae and areola mammae, the papillae are very conspicuous in formation against the epidermis, the development of the sensory fibres termi-

nating here is also very good. On the contrary, the common haired skin is so poorly provided with papillae, that almost no formation of papillae is generally observable, and the development of the sensory fibres is also very slight in these parts.

The terminal territory of the so-called sensory hair nerve fibres ending in connection with hairs is represented by the Seto's hair neuroshield or -tube composed of special tissue filling the hair follicle neck. The neuroshield is formed on one side of the hair root sheath, while the neurotube is formed multilaterally, encircling the sheath. Accordingly, the development of the sensory nerve terminations in the latter is stronger than that in the former. As a multilateral development of sebaceous glands is necessary for the formation of the hair neurotube, it follows that for the development of the hair sensory nerve fibres, a good development of the sebaceous glands is prerequisite.—It goes without saying that the hair sensory nerve fibres are proportional in mass to the density of hair growth.

In the back skin I studied, the epidermis is somewhat thicker than in the eyelid, the pars cutanea of lip and the nose, being roughly equal to that of the scalp. Some small papillae are also here formed from the connective issue of the papillar layer of the corium, but they are far inferior to those in the external genitals etc., in development. According to the law enunciated by Seto to the effect that the development of sensory fibees is in direct ratio with that of the papillae, the papillar layer of the back skin must be poor in sensory nerve fibres as in other parts of the haired skin. In fact, I found the papillar layer of the back skin penetrated by very few sensory fibres ending in very simple terminations, as are the cases with the scalp (Seto)[1], the eyelid (Seto)[2], the pars cutanea of lip (Seto *et al.*)[3] and the nose and the pars cutanea of vestibulum nasi (Goto, yet to be published).

Just as in the scalp (Seto[1]), so in the back skin, the nerve bundles consisting of sensory and vegetative fibres penetrating the subcutaneous tissue spread out in the sweat gland layer to pass over into the comparatively coarse meshed plexus. From this plexus emerge sporadically small nerve bundles, which advance into the reticular layer of the corium. Almost all the sensory fibres in each bundle run into the definite special terminal territory in the follicle neck to end therein, only a very small number of them penetrating further into the papillar layer to terminate. The terminal formation of the latter is very simple, that is, represented by unbranched sharp endings in most cases but in rarer cases by simple bifurcated sharp endings derived from medullated fibres running into the papillar layer and losing their myelin, as shown in Figs. 1 and 2. Anyhow, the number of such sensory fibres is extremely small, and as the back skin is also of incessantly desquamating nature, no intraepithelial fibres are found therein, in conformance with Seto's reported observations[1] on such skins.

The reticular layer in the back skin is of a very great thickness and

Fig. 1

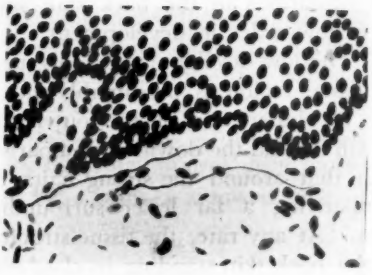

Fig. 2

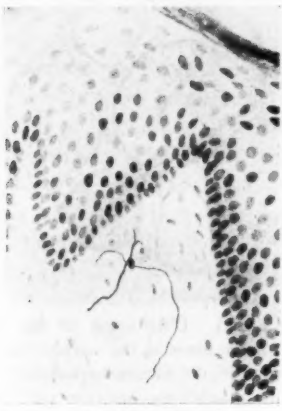

Fig. 1. Unbranched sharp endings found in the subepidermal layer of the human back skin. Seto's silber method, X 300, reduced to ½).

Fig. 2. An unbranched and a bifurcated ending found in the same place. Same staining, X 300, reduced to 2/3.

is separated from the adipose subcutaneous tissue by the sweat gland layer. Now the hairs rising from this reticular layer which are of utmost importance in respect to the sensory innervation of the back skin, are very sporadic in growth, as may be readily observed ocularly, which fact is sufficient to prove the poor development of sensory fibres in the back skin.

What attracted my special attention concerning the hairs on the back skin was the fact that they had their origin in the deeper layer of the thick corium and sometimes even in the subcutaneous tissue, unlike the downs on the face etc., so that the hair roots and follicles are of great length comparable with those of the hairs on the head, and also the fact that the sebaceous glands show a very specific development, that is, they are not developed powerfully all around each hair follicle, as is the case with the vibrissae and the hairs on the pars cutanea of lip, but stretch down far into the deeper layer of the corium along the hair follicle, sometimes even down into the sweat gland layer. In most cases, 2–4 gland bodies are developed on one side of the hair follicle, as shown in Fig. 3, but on rarer occasions, they show multilateral development and surround the hair follicle completely on every side.

What is of further interest is the fact that, as shown in Fig. 3, the follicle neck formed between the sebaceous glands and the outer root sheath are very ill-developed and narrow, so that the terminal area of the hair nerve fibres represented by the annular accumulation of spindle-shaped special cells in the follicle neck, that is, the Seto's hair neuroshield

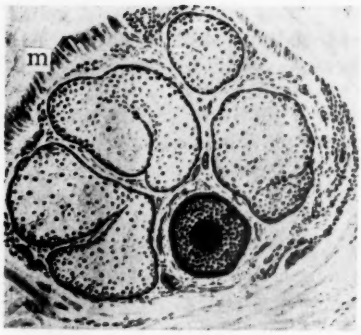

Fig. 3. Distribution of the sensory hair nerve fibres in the narrow hair neuroshield in the human back skin. s sebaceous gland; m arrector pili muscle. Same staining, X 120, reduced to 2/3.

or -tube, is also very poorly developed. It naturally follows that the sensory hair nerve fibres as well as their terminal formations in the back skin must be of low order in development.

The low development of this specific tissue region is presumably due to the far lesser size of the outer root sheath around the downs in comparison with that around the strong hairs, accompanying a far lesser surrounding area. At any rate, the tissue structure of the back skin standing in close relation with the sesnrory supply is very characteristic in that the downs originate in the deeper layer of the corium and grow very sporadically, the sebaceous glands are elongated and strongly developed and that the neuroshields and -tubes where the sensory hair nerve fibres end are very poor in formation.

As described above, the sensory nerve fibres supplying the back skin are very lowly in development, but the majority of the sensory fibres penetrated into the hair neuroshield or -tube through the bottom of the follicle neck. However, as these terminal areas are very low in development, the terminations of the sensory fibres must be also of simple formation in general. In fact these terminations in the back skin are much simpler in formation than those in the hairs of the head, which are rather simple in structure, but less simple than those for the downs on the eyelids.

The terminations of the sensory hair nerve fibres on the back skin are classifiable in general as follows: 1. Plexus-like terminations (Fig. 4): Several sensory fibres, upon penetrating into the terminal territory, ascend in somewhat mutually divergent courses, frequently sending out branches and intercrossing themselves in the courses, show a plexus- or bush-like arrangment in general appearance. The terminal branches end sharply or bluntly in most cases, but sometimes they terminate in nodes or bulbs, and are apparently in very close relation with the special cells composing the terminal territory. The terminal branches frequently penetrate into the cell bodies, to end in contact with the cell nuclei. Such terminal conditions are not limited to the plexus-like terminations but are also common to other terminal formations described below. The plexus-like terminations are much simpler than those in the scalp, and even incomparably simpler than the plexus-like terminations found around the

cilia and the downs in the pars cutanea of lip.

2. Serrate terminations: In these terminations, the sensory fibres, upon entering the terminal territory, bifurcate and run into the lower projections of the nerve elements arranged in serration. From the upper projections nerve fibres are further sent out, which soon come to sharp ending. Terminations of this type are found only in a very small number in the back skin, since they belong to the more complex type of hair nerve terminations. Around the cilia and the downs in the pars cutanea of lip, these complex terminations are found in a large number, but in the back skin, they appear even more rarely than in the scalp.

Fig. 5 shows a mixed type of plexus-like and serrate terminations.

Fig. 4

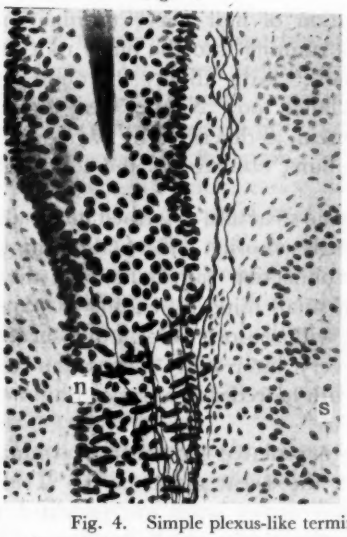

Fig. 5

Fig. 4. Simple plexus-like termination of sensory hair nerve fibres found in a hair neuroshield. Human back skin. n specific cell nuclei in the neuroshield; s sebaceous gland. Same staining, X 3000, reduced to $\frac{1}{4}$.

Fig. 5. A mixed type of plexus-like termination and serrate termination of the sensory hair nerve fibres formed outside the outer root sheath showing a remarkable tendency to atrophy. Human back skin. n specific cell nuclei in the hair neuroshield; s sebaceous gland. Same staining, X 300, reduced to $\frac{1}{2}$.

Here, the termination is composed of a comparatively large number of sensory fibres, representing a rather complex formation, but such complex terminal formation is of very rare occurrence in the back skin. Moreover, this termination of mixed type has been formed outside the outer

root sheath which shows a remarkable tendency to atrophy upon falling off of the hair, so that the nerve elements have been congested in a small space and show the appearance of a complex termination.

3. Indefinite type terminations: As Seto[1] has described such terminations in the scalp, in these terminations, 2–3 rather thick sensory fibres, upon entering the terminal territory, run very irregular courses, sending out frequent branches, which also run irregular courses, all ending sharply, bluntly, in nodes and other terminal formations of varying description. The fibres sometimes show remarkable change in size in the course. As these terminations represent simple terminal formation of hair nerve fibres in essence, such terminations are almost never found with cilia and the downs in the pars cutanea of lip, where nerve fibres are very richly provided.

Finally, throughout the examination of hair nerve terminations, I failed to find any intraepithelial fibres running into the outer root sheath to end there, as described by Kadanoff,[7] with all my thorough search for them, as Seto and his collaborators failed too. Neither were the tactile cells and the nerve elements ending therein, as described by Szymonowicz[8] and Kadanoff[7] as found in the epithelium, observed in the course of my study.

As may be conjectured from the tissue structure described in the above, the back skin is very poorly innervated sensorily. Here also, the great majority of the sensory fibres ends in connection with hairs, as is the case with all haired skin in general, only a small minority ending in papillae. Thus the back skin is very ill provided with sensory fibres, but I succeeded in proving therein the existence of very interesting corpuscular terminations, never reported in connection with haired skin as far as I am aware, though indeed in very rare cases.

The first type of them consists in capsulated glomerular termination formed along the small artery wall in the sweat gland layer. Truly, it has been known from earlier days that artery walls are provided with sensory terminations, but only a comparatively small number of such innervated arteries are known to date, namely, the arcus aortae, the cardiac arteries, the sinus caroticus, the brain arteries and the pial arteries.

This terminal body is oval and rather large in size as shown in Fig. 6 and is found in contact with the wall of a small artery. It is covered by a thin connective tissue capsule and is filled with granular ground substance, the so-called inner bulb, containing many round or elliptical special nuclei. Into the inner bulb rather thick medullated fibres are seen penetrating, after loss of myelin, and branching out to an extent therein. The branches run peculiar ansiform courses, showing a general appearance of somewhat complex glomerulus. The nerve elements in

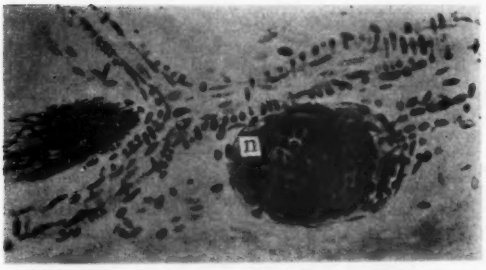

Fig. 6. A capsulated glomerular termination found in a small artery wall in the sweat gland layer of the human back skin. n specific cell nuclei in the inner bulb. Same staining, X 300, reduced to $\frac{1}{3}$.

the inner bulb show remarkable change in size, come into frequent anastomosis and the terminal branches come to sharp, blut or sometimes bulb-like endings. The nerve bundle appearing on the right side of the picture composes a nerve plexus consisting of numerous fine vegetative fibres and one very stout sensory fibre, which seems to represent the trunk fibre to the glomerular termination.

The glomerular termination described above is of more complex formation than that described by Hirsch[9] in the femoral artery. The fact that such glomerular endings were ever found in the small artery wall in the subcutaneous tissue of the back skin is decidedly of deep interest, from the view point of their physiological interpretation alone. It is beyond doubt that these bodies are sensory terminations connected with the arteries, seeing from their location. It seems that they are receptor apparatus related to the blood circulation, in particular, to the adjustment of the blood pressure.

The second type of the corpuscular terminations consists in the terminations I discovered in the papillary layer of the back skin. I need not repeat that genital nerve corpuscles and minor Pacinian bodies are found in the papilla mammae and areola mammae, and in the external genitals, that is, in the scrotum and the praeputium etc., but such bodies have never been reported in any other part of the haired skin. So, my discovery of rather complex non-capsulated glomerular terminations in the papillae of the back skin may be called an extremely interesting achievement, though of course the number of such glomeruli is very small.

As shown in Fig. 7, a thick sensory fibre, having ascended into the papillar layer and lost the myelin, repeatedly sends out branches, which end in a glomerular arrangment after ansiform courses accompanying marked change in size. This glomerular termination made of such nerve fibres is also formed in a ground substance containing specific nuclei,

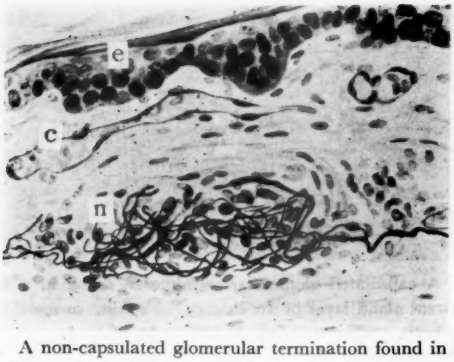

Fig. 7. A non-capsulated glomerular termination found in the papillar layer of the human back skin. e epidermis; c blood capillaries; n specific cell nuclei in the inner bulb. Same staining, X 400, reduced to 2/3.

alike the capsulated glomerular termination found in the artery wall described above. It seems no connective tissue capsule is formed here.

According to Seto and his disciples, the corpuscular terminations, especially, the glomerular terminations, presumably represent receptor apparatus for very delicate stimuli. I am in concurrence with such an opinion.

Upon the distribution of the vegetative nerve fibres in the haired skin, Seto has reported very minutely in his study on the scalp. In my back skin, as would be expected from what Seto[7] has reported, I found nerve bundles composed of fine vegetative fibres and thick sensory fibres running into the cutis, which pass into the nerve plexus formed in the sweat gland layer, as related above. In the sweat gland layer, the vegetative nerve branches emerging from the plexus spread out in minute trami toward the sweat glands, finally to pass over into the vegetative terminalreticulum dissolved around the sweat glands.

The vegetative fibres in the hair nerves that run into the hair follicle neck together with the sensory fibres form also the terminalreticulum around the outer root sheath, which comes in contact with the blood capillaries and the outer root sheath and enters into controlling relation over them. Otherwise, the vegetative nerve terminations are conspicuously formed for the arrector pili muscles and the connective tissue in the papillary layer too.

For example, Fig. 8 shows a vegetative terminalreticulum spread out in the papillary layer. It stands in tactile control over the blood capillaries, the connective tissue cells and the basal layer of the epidermis in this part. Besides, vegetative nerve fibres form the perivascular plexus along the adventitia of blood vessels, especially, of arteries, which gradual-

ly passes over into the terminalreticulum toward the tunica media.

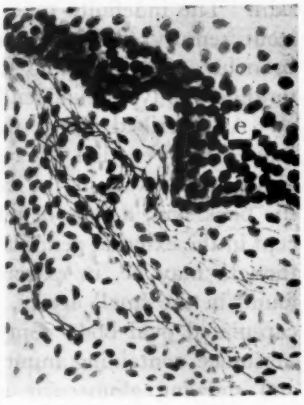

Fig. 8. The vegetative terminalreticulum distributed in the papillar layer of the human back skin. e epidermis. Same staining, X 400, reduced to ½.

Summary

The poor sensory innervation of the human back skin between the 5th and the 8th thoracal spinal processes may be surmised from the peculiar histological picture of this part, which shows very sporadic growth of hairs, small terminal territory of the sensory hair nerve fibres in the hair follicle neck and a weak development of the subepidermal papillae, because, as Seto and his collaborators have established, the majority of sensory fibres in the haired skin terminates in connection with the hairs and the minority that comes into the papillary layer is developed in proportion to the development of the papillae.

In fact, the sensory fibres running into the papillar layer were very small in number, and their terminations were very simple, being represented by sharp unbranched, and in rarer cases, by bifurcated sharp endings. No intraepithelial fibres were ever found.

The hairs in this region are downy, but they reach far into the deeper layer of the corium, together with the much elongated sebaceous glands. The terminal areas of the sensory hair nerve fibres, the Seto's hair neuroshields or -bubes formed between the sebaceous glands and the outer root sheathes are generally very poorly formed, and consequently the development of the sensory nerve fibres and their terminations is also extremely weak. The sensory terminations are even simpler than those in the scalp (Seto)[1], but more complex than those with the downs on the eyelids (Seto[2]).

The terminal formation is entirely of simple plexus-like, serrate and indefinite types. The plexus-like terminations are formed by sensory fibres and their branches arranged in plexus form, and are incomparatively simpler in formation than those with cilia (Seto[2]) and the downs of the pars cutanea of lip (Seto *et al.*[3]) and even simpler than those in the scalp (Seto[1]).

The serrate terminations are composed of sensory fibres arranged in serration, but since these belong to the more complex type of hair nerve terminations, only a very few of them are found in the back

skin. The indefinite type of termination is represented by a few rather stout sensory fibres running an irregular course, together with their branches, and shows no definite terminal formation. Being of simpler termination type, such endings are frequently found in the back skin.

As described above, the back skin is very poor in sensory fibres, but interestingly enough, there were found glomerular terminations in the small artery wall in the subcutaneous tissue and the papillary layer of the back skin, a type of terminations never found in the haired skin except in the external genitals and the papilla mammae and areola mammae, though, may be, in extremely rare cases. This glomerular termination found in the small artery, but is more complex. It is represented by a capsulated oval body composed of sensory fibres running into a ground substance containing numerous cell nuclei together with a few branches and showing glomerular arrangement, and is presumably receptor apparatus connected with adjustment of blood pressure. The other corpuscular body in the papillar layer is composed of non-capsulated glomerular ending formed in the same ground substance, and is perhaps receptor apparatus for delicate stimuli.

The vegetative innervation of the back skin is much the same with that of the scalp (Seto)[1].

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Fine Structure of Nasal and Oral Cavities in Dog and their Sensory Innervation, especially on the Intraepithelial Fibres

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(Received for publication, September 12, 1953)

The fact that, in the alimentary canal in man, its entrance, the oral cavity, and its exit, the anus, and in the respiratory tract, its entrance, the vestibulum nasi, are rich in nerves, especially in sensory nerves, has been clearly demonstrated by many studies in this laboratory. The terminations of these sensory fibres are chiefly formed in the tunica propria of the mucous membrane, especially in the papillae, but in places, the sensory fibres penetrate further into the epithelium, to end there as intraepithelial fibres.

There have been reported many studies on these sensory terminations in the past, but owing to the extreme difficulty of adequately staining nerve fibres, there seems to exist many points of dubious validity in these studies, in particular, on the intraepithelial fibres. For example, intraepithelial fibres have been found in parts where no such fibres had to exist, such as the epidermis of haired skin or the outer follicle sheath, and even in the parts where intraepithelial fibres might be expected, the delineated intraepithelial fibres represented nothing but illusory artefacts. Such unreliable informations were rather regrettably frequent in the past literature.

Since about 20 years ago Prof. Seto and his many co- workers in this laboratory have studied on the sensory terminations in the above mentioned propria as well as the epithelium, with human mucosa as objective, so that the research on this subject seems to have reached a stage of final completion.

These studies have elucidated, among other things, that intraepithelial fibres are never formed in the incessantly desquamating cornified stratified flat epithelium, such as the epidermis of haired skin, and that they are found only in limited areas of the non-cornified stratified flat epithelium, for instance in vestibulum nasi, transitional part of lips, —gums, hard palate and anus. They are also found in the ciliated epithelium, but again in limited areas, namely, in larynx, pharynx, trachea and

bronchi, but not in cavum nasi. In the stratified columnar epithelium they are found in urethra, and in the transitional epithelium, for example in vesica urinaria and urethra. Besides, in the taste buds and in the common epithelium between glans penis or glans clitoridis and the inner plate of praeputium in embryonic time, conspicuous intraepithelial fibres are observable.

The nerve fibres penetrating into the epithelium are never vegetative, but always sensory in nature, so that it may be readily surmised that the areas rich in such fibres are extremely sensitive. The character and the formation of the intraepithelial fibres are more or less different according to the areas of their existence.

As described above, the knowledge on the sensory terminations in the mucous membrane has been greatly amplified by such studies in this laboratory. The author of this report has also been granted the opportunity of succeeding them in their study, with the sensory terminations, especially the intraepithelial fibres, in the mucosa of the nasal cavity with the snout and the oral cavity in dog, one month of age, as my objective. My materials were fixed for a long time in 10% neutral formol, cut into 30–40μ frontal and sagittal frozen sections, and stained with Seto's silver impregnation. The series of the very beautifully stained preparations thus obtained I subjected to a minute examination and arrived at the observations reported in the following.

Individual Observations

Innervation of mammalian nasal cavity:

I could not find any literature on this subject, while on the innervation of the human vestibulum nasi and pars respiratoria of cavum nasi, Seto and Sada[1] (1944) and Sato[2] (1949) have respectively published detailed and dependable studies. According to Sato,[2] in the subepithelial connective tissue of pars respiratoria of human nasal cavity there are found numerous vegetative nerve fibres, but only a few of sensory fibres originating from the trigeminal nerve. The terminations of these latter fibres are of the simplest formation, and are located mostly in the subepithelial propria, almost none of them penetrating into the epithelium itself.

In the mucosa of the human vestibulum nasi (Seto and Sada)[1], however, we can find beside branched terminations also corpusclar terminations in the propria, especially in its papillae and further intraepithelial fibres in many places. Since the distribution of sensory fibres generally stands in close relation with the minute structure of the mucous membrane, Seto and Sada also here in vestibulum nasi conducted a minute research on its fine structure, especially on the epithelium and the papilla formation, and in accordance with many studies in this laboratory, proved that the more remarkable the development of the papillae, the better is the sensory supply here too.

The human vestibulum nasi is divisible into pars cutanea with vibrissae and pars mucosa, and the latter again into the corneous and the non-corneous parts. Besides, a narrow transitional zone covered with stratified columnar epithelium lies between the vestibulum and pars respiratoria of cavum nasi. In the corneous mucosa part, the epithelium is much thicker than that in pars cutanea, and is composed of a stratified flat epithelium provided with corneous plate instead of stratum corneum. Samall papillae formed by the subepithelial connective tissue are arranged regularly against the epithelium. The non-corneous mucosa part is covered by a stratified flat epithelium without corneous plate, and the papilla formation is here so very poor, that the basal surface of the epithelium becomes almost smooth and even upon approaching the transitional zone.

The development of the sensory fibres is especially marked in the corneous mucosa part where the papillae are also in advanced formation, and corpusclar terminations and intraepithelial fibres of considerable size are found existing. In the non-corneous mucosa part, sensory fibres are much poorer in development, but the formation of minute branched terminations consisting of a few rami is often found in the epithelium.

Now, the results of my studies upon the minute structure of the mucous membrane of the canine nasal cavity are in good accordance with those in man. The mucous membrane of pars respiratoria of cavum nasi in dog is lined with stratified ciliated epithelium, nasal glands and well-developed venous plexus are found in the tunica propria and submucosa which are almost indivisible from each other, but the smooth muscle fibres found in human cavity are not found here. In this respiratory part there are found rather thick nerve bundles running through the submucosa, mostly composed of minute non-medullated vegetative fibres, which gradually branch out and anastomose mutually, finally to pass into the terminalreticulum and to distribute around the blood vessels. On the contrary, the sensory fibres found in the nerve bundles are much smaller in number. They run into the tunica propria directly beneath the epithelium and end there as unbranched or simple branched terminations. No fibres have ever been found to penetrate further into the epithelium.

The fine structure of vestibulum nasi in dog is much the same with that in man, but some interesting differences are found in that the canine vestibulum nasi lacks pars cutanea with vibrissae, and its front part passes partly into the suout mentioned below, partly into the haired skin. The distribution area of the corneous and the non-corneous mucosa parts is very varied according to the position, but generally speaking, the vestibulum seems to be almost divided by both the mucosa parts into the anterior and the posterior halves.

The epithelium in the corneous mucosa part is much thicker than the epidermis of the haired skin, consisting of stratified flat epithelium covered by corneous plate. The propria forms low, small papillae arranged regularly against the basis of the epithelium. The non-corneous mucosa part is covered by a stratified flat epithelium without corneous plate, the basis of which grows gradually smoother as the deeper part is approached. It must be specially

mentioned that this vestibulum mucosa has much poorer developed nasal glands than in man, which are distributed very sporadically, and weakly developed venous plexus.

Also in canine cavum nasi, much more nerve bundles are found penetrating the submucosa of the vestibulum nasi than that of the respiratory part. These bundles contain a large number of thick medullated sensory fibres beside the thin vegetative fibres. The bundles gradually branch out and the vegetative fibres therein finally pass into the terminalreticulum, chiefly spreading out in the tunica propria, while the sensory fibres run into the corneous, rather than into the non-corneous, mucosa part in a far greater number, to end mostly in branched terminations in the subepithelial connective tissue, especially in the papillae. Such corpusclar terminations, as found by Seto and Sada[1] in human vestibulum were not to be seen in my baby dogs. But when we consider that the corpusclar endings, excepting the Pacinian bodies, are yet unformed in human embryos, it may be supposed that such bodies are formed later on also in dog, gradually as it grows to maturity.

Seto and Sada[1] have found a comparatively large number of intraepithelial fibres in the epithelium of human vestibulum, and classified them into branched terminations composed of minute fibres and short unbranched terminations consisting of thick fibres coming in from the top of papillae and reaching the upper layer of the epithelium. Also in my dog vestibulum, I found intraepithelial fibres even in a larger number than in man, especially numerous at the entrance of the vestibulum. In outer skin covered by desquamating corneous epithelium, no intraepithelial fibre is ever found, but it is of interest that such fibres appear as soon as we pass from the haired skin into the vestibulum.

Some of these intraepithelial fibres originate in the branched terminations formed in the papillae (Figs. 1 and 2), and others are represented by sensory fibres running singly through the papillae and penetrating directly into the epithelium. Some of the latter end in unbranched terminations without branching out (Fig. 3), while others end in branched terminations after ramifying in the epithelium (Fig. 4). These fibres are in most cases smooth-surface, thin and uniform in size throughout, but some are thicker, as shown in Fig. 2. They run through or between epithelial cells and end sharply or more rarely in small nodes (Fig. 3) in any of its layers. No such short but very stout unbranched intraepithelial fibres running out of the top of papillae, as found by Seto and Sada[1] in human vestibulum nasi, was ever found in my baby dogs.

Innervation of the snout: The snout, peculiar to the mammals, is very rich in sensory nerve fibres, and having particularly well-developed intraepithelial fibres, has offered an appropriate theme for many investi-

Fig. 1

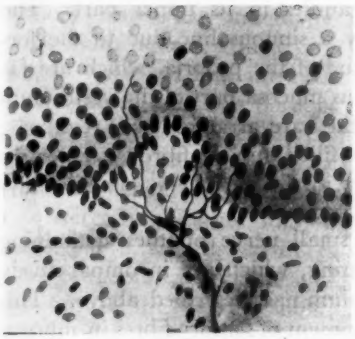

Fig. 2

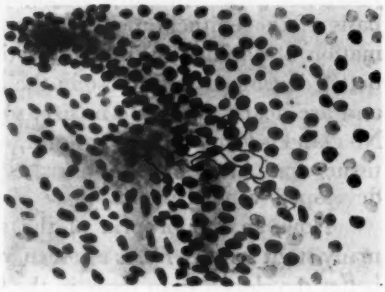

Fig. 1. Branched sensory termination formed in a papilla in the corneous mucosa part near the entrance of the vestibulum nasi in dog. Seto's impregnation, X 600, reduced to ½.

Fig. 2. Ditto. The terminal fibres are of comparatively thick nature. Same staining, X 600, reduced to ½.

Fig. 3

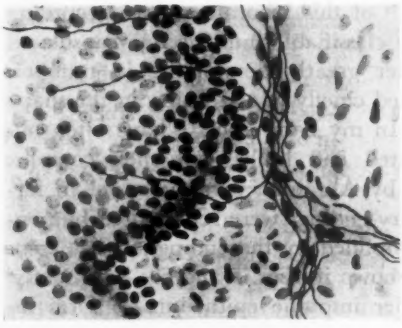

Fig. 4

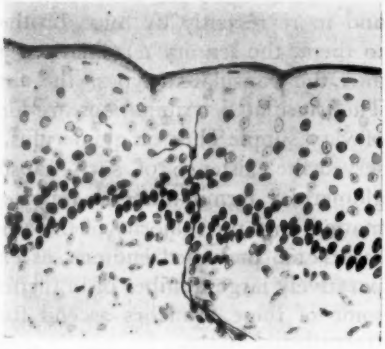

Fig. 3. Unbranched intraepithelial fibres ending sharply or in node originated in singly running sensory fibres seen in the corneous mucosa part of vestibulum nasi in dog. Same staining, X 400, reduced to ½.

Fig. 4. Bifurcated intraepithelial fibre found in the corneous mucosa part of vestibulum nasi in dog. Same staining, X 400, reduced to ½.

gators in the past. I will preface my description on the innervation of this part with a histological picture thereof, because it has a close bearing on the nerve distribution. The snout is presumed to represent the foremost part of vestibulum nasi from the ontogenetic point of view. In fact, the epithelium thereof is very similar to that of the corneous mucosa part of the vestibulum, consisting of stratified flat epithelium having corneous

plate, somewhat larger in height than that in the vestibulum and containing a large quantity of pigment granules in its upper part. The basis of the epithelium is represented by a sinuous line due to the formation of papillae by the connective tissue of the propria, which are far better developed than those in the corneous mucosa part of the vestibulum and are nearly the same in size, so that they are arranged very regularly. No gland is formed in the propria, which is filled up with fibrous connective tissue, containing in some parts, especially at the periphery, some weakly-developed venous plexus.

Now, in the propria of the snout, small nerve bundles containing many stout sensory fibres are seen penetrating, which are incomparatively better developed than those in the vestibulum nasi described above. This fact again proves the thesis that the development of sensory fibres in mucous membrane covered by stratified flat epithelium is in proportion to the development of papillae, as has been established by many researches completed in this laboratory.

These sensory fibres run into the tunica propria, in particular, into the papillae, to end there chiefly in branched terminations. The sensory nerve distribution in adult dog has been studied by Ábrahám[3] (1931) and more recently by Suga brothers[4] of this laboratory, and according to them, the sensory terminations are classifiable into the non-capsulated and the capsulated types, the former greatly dominating in number. The capsulated terminations are formed chiefly of end bulbs, in the shape of very simple club or glomerulus. In my baby dogs, I have often observed the existence of such capsulated end bulbs, but such complex glomerular terminations as reported by Ábrahám[3] were not yet in formation, and the majority of the sensory endings were represented by non-capsulated branched endings, as exemplified by the endings with a comparatively large number of branches shown in Figs. 5 and 6. Frequently, some of these branches ascend further into the epithelium to form intraepithelial fibres.

The intraepithelial fibres in mammalian snouts have been the objectives of many past studies by Szymonowicz,[5] Trejakoff,[6] Merkel,[7] Botezat[8] Kadanoff,[9] Boeke,[10] Akkeringa,[11] Hashizume,[12] Huss,[13] Cybulsky,[14] Momono and Tsuda,[15] but the majority of these studies were made on pigs, succeeded by studies on moles, cows and rabbits, only few, such as by Ábrahám[3] in older times and by Suga brothers[1] recently, being conducted with dogs as objectives. Botezat[8] was successful in pointing out the existence of intraepithelial fibres in his methylene-blue stained preparations, but owing to the imperfection of his staining method, has missed to bring out the true nature of intraepithelial fibres. The results obtained by Ábrahám[3] by use of silver method have been

more commendable, but the sensory fibres are much too thick in his figures. Suga brothers[4] failed to find intraepithelial fibres. This is perhaps due to their examining only the upper part of the snout rich in pigment granules.

According to my studies, numerous intraepithelial fibres were found to exist in the epithelium of the canine snout, which are oftener branched than unbranched at that. These intraepithelial fibres include those represented by the rami of the branched terminations formed in the papillae penetirating further into the epithelium (Figs. 5 and 6), and those originating in single fibres running into the epithelium and branching out there (Figs. 7, 8 and 9), as was the case in the vestibulum nasi.

These intraepithelial fibres are either thin (Fig. 8) or comparatively thick in nature (Figs. 5, 6 and 7), as in the mucosa part of the vestibulum, but I could not find any such stout fibre as reported by Ábrahám.[3] The fibres run through or between the epithelial cells and end sharply, or sometimes in small nodes, in the upper layer of the epithelium. However, it is not at all rare that they terminate in the basal or the middle layer of the epithelium.

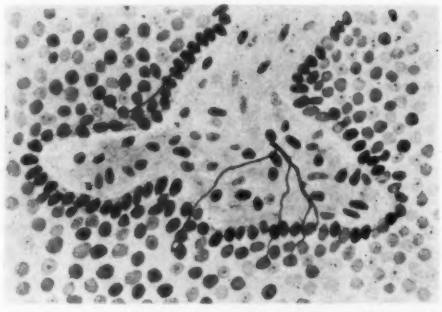

Fig. 5. Branched termination consisting of comparatively thick fibres seen in a papilla in the snout in dog. Its terminal fibres advance into the epithelium, to end sharply or in node. Same staining, X 600, reduced to ½.

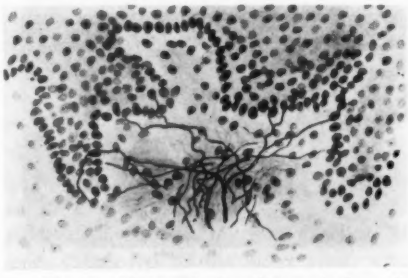

Fig. 6. Unbranched and branched terminations consisting of somewhat thick fibres seen in a papilla of the snout in dog. Most of the terminal fibres enter furthermore into the epithelium. Thin fibres are vegetative nerve fibres. Same staining, X 200, reduced to 2/3.

Of the innervation of the mucous membrane of the canine oral cavity, Okano[16] of this laboratory has previously reported in detail on his study on its lingual mucosa, so I will report only on the other parts, that is, on the lips, the cheeks, the gums and the palate mucosa. The studies hitherto reported on the innervation of the dog oral cavity have been centered on the tongue and the hard palate, but only few of them touching

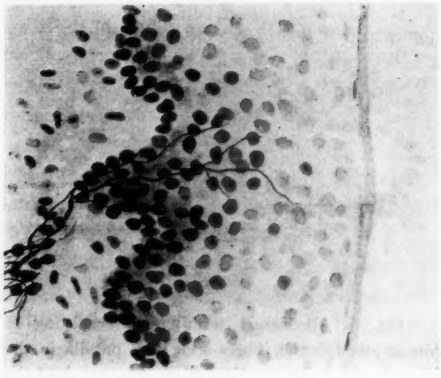

Fig. 7. Branched intraepithelial sensory termination consisting of somewhat thick fibres seen in the snout in dog. All of the terminal fibres end sharply. Same staining, X 600, reduced to ½.

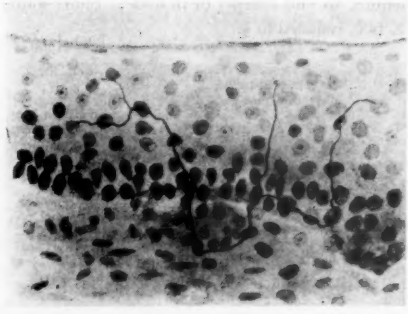

Fig. 8. 2 bifurcated intraepithelial fibres seen in the snout in dog. Same staining, X 600, reduced to 2/3.

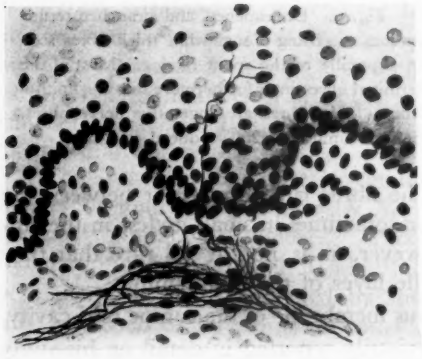

Fig. 9. Branched intraepithelial fibre seen in the snout in dog. The terminal fibres end sharply. Same staining, X 600, reduced to ½.

the lips, the gums and the cheeks.

Nerve supply of lips and cheeks in dog:

Upon this subject, we have had no investigations with mammals as objectives, but there have been the brilliant studies by Fujii[17] and Suga[18] of this

laboratory respectively conducted with human adults and embryos. The human lips, as reported by Luschca,[19] Neustätter[20] and others, consist of the pars cutanea, the pars transitionis and the pars mucosa. The transitional part represents the part lying between the outer skin part and the inner mucosa part and are divided into the inner and the outer zones by a conspicuous furrow in the latter stage of embryonic life. The inner zone, due to the formation of lip downs on its surface, has been called the pars villosa and the outer zone, the pars glabra, as it remains smooth (Luschca).

As in the lips also, the development of sensory fibres goes with the development of the papillae, the histology of this part has been studied by Fujii[17] and Suga[18] very minutely. Such studies have revealed that the epithelium suddenly increases in height as it goes over from the pars cutanea to the outer zone of the transitional part, directly beneath which regularly aligned small papillae are formed, which are not observable in pars cutanea. Upon reaching the inner zone, the epithelium is rapidly thickened again, and the papillae become also more powerful. However, in pars mucosa, both the height of the epithelium and the development of the papillae become considerably poorer. The epithelium is composed of stratified flat epithelium, with corneous plate in the outer, and non-corneous in the inner, zones. This histological picture is especially vivid in the latter stage of human embryonic life, but that in adult lips also shows a glimpse of such a genesis, that is, there is no essential histological difference between the embryonic and adult lips.

The development of the sensory fibres in human lips is most emphasized in the inner zone of the transitional part, next in the outer zone thereof, the mucosa part standing in an inferior position to the two foregoing in this respect. Thus, the sensory terminations are best developed in the inner zone where the papillae are also most strongly developed, succeeded by the outer zone, where the papillae are in good formation, in spite of the low epithelium, but is poor in the mucosa part, where the epithelium is rather thick but the papillae are not so well developed.

The sensory terminations in the lips are chiefly of branched type in the outer zone, but also some non-capsulated glomerular endings, or endings resembling the genital nerve bodies are found existing here. Intraepithelial fibres originating in these endings are also found in many places. In the inner zone, the sensory fibres are better developed, their endings consisting of more complex branched and glomerular endings, accompanying a small quantity of intraepithelial fibres. In the mucosa part, however, there are found some simpler glomerular and quasi-genital bodies, but no intraepithelial fibres. In human embryo, no corpusclar termination is yet formed in the lips.

The histological picture of the lips of my baby dogs resembles strontly that in human embryo. The pars cutanea consists of a corium with numerous hair follicles and a thin corneous epidermis without papillae. Interestingly enough, the canine lips are also divided into the inner and outer zones at the transitional part, by a shallow furrow, as is the case with human lips. The outer zone occupies only a small space, and its epithelium is about 3 times that

in the pars cutanea in thickness, is composed of a stratified flat epithelium with corneous plate, and considerably well-developed papillae are formed against it. Upon going over from the outer to the inner zone, the epithelium grows promptly in thickness, reaching 4 or 5 times of the thickness in the outer zone at the middle part of the inner zone. The epithelium consists of non-corneous stratified flat epithelium without corneous plate, and villi-like epithelial cell scales, as seen in human embryo, are found on its surface. The formation of papillae becomes stronger, especially increasing in height. This part occupies a far larger space than the outer zone, and the height of the epithelium grows gradually thinner as it goes over into the mucosa part. In the latter part, the epithelium decreases in height, so that it becomes as low as in the outer zone. In contradistinction to the human counterpart, there is little formation of papillae. In the cheek mucosa lying exterior to the lips, the epithelium is high and provided with well-developed papillae in the part above the level corresponding to the rima oris, as in human cheek, but in other parts, no papillae are formed and the epithelium is also much lower.

As the histology of the mucous membrane of the lips and cheeks in dog in its similarity to that in man clearly reveals, the sensory supply of these parts is also much the same in man and dog. So, the inner zone of the transitional part, the outer zone thereof and the mucosa part of the lips are best supplied, in the order named. However, the number and the terminal formation of the sensory fibres are much inferior in dog lips to those in man. Similar inferiority has been demonstrated in dog tongue by Okano,[11] too. In the cheek mucosa, the parts above the level corresponding to the rima oris provided with papillae are rich in sensory fibres, but the other parts are very poorly supplied.

The sensory fibres in the transitional part of the canine lips, both in the outer and inner zones, end in papillae, generally in comparatively simple branched endings, but sometimes also in simplest unbranched endings, no such complex branched and corpusclar endings as found in man being observable. Intraepithelial fibres are found in the outer zone, consisting mostly in thin smooth-surfaced fibres, often sending out rami (Fig. 10), and quite as often running out unbranched, to end sharply or in small nodes in the upper layer of the epithelium.

Innervation of hard palate in dog: There have been many studies on the nerve supply of the hard palates of mammals as cited in the report on that in cat and hedgehog by us,[21] but these studies are of rather dubious value owing to the imperfection of staining methods. So, we have made a research on the same question of the two kinds of mammals mentioned above, utilizing the Seto's impregnation, and similar to those in man in distribution, but as much as the palates of these animals show histological pictures simpler than in man, the number and terminal formation of

sensory fibres were also poorer. The majority of these sensory terminations in the papillae were unbranched, as well as the intraepithelial fibres.

The sensory fibres in the hard palates of my baby dogs also were poorer than in man, both in number and in terminal formation, as was the case in cat and hedgehog, but somewhat better than in cat in both respects. In cat, the large majority of the sensory terminations were merely simple unbranched endings, but in dog, the terminations often consisted in more complex branched endings, as shown in Fig. 11. Intraepithelial fibres

Fig. 10

Fig. 11

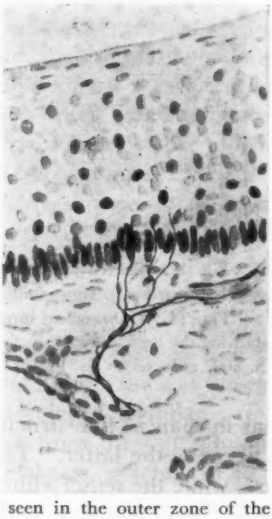

Fig. 10. Branched intraepithelial fibre seen in the outer zone of the transitional part of the dog lip. Its running course through and between the epithelial cells is seen clearly. Same staining, X 500, reduced to 2/3.

Fig. 11. Somewhat complex branched termination formed beneath the epithelium of the dog hard palate. Most of the terminal branches enter the basal layer of the epithelium, to end sharply. Same staining, X 400, reduced to 2/3.

were more numerous in dog and they were not always represented by simple unbranched terminations (Fig. 12) as in cat but often by branched endings (Fig. 13). These also consisted of uniformly thin smooth-surfaced fibres running through or between the epithelial cells and ending sharply or in small nodes in the upper layer of the epithelium, as was the case in cat and hedgehog.

Summary

The mucous membrane of the canine nasal cavity is nearly the same

Fig. 12

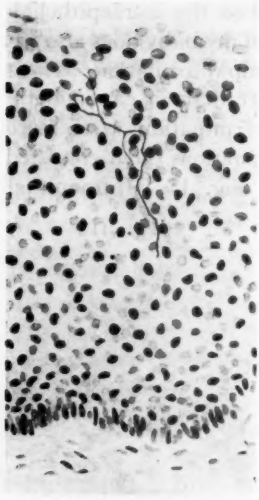

Fig. 13

Fig. 12. Somewhat complex branched intraepithelial fibre seen in the dog hard palate. Same staining, X 300, reduced to $\frac{1}{4}$.

Fig. 13. Unbranched intraepithelial fibres seen in the dog hard palate, showing the typical smoothsurfaced and uniformly thin fibres. Same staining, X 300, reduced to $\frac{1}{4}$.

with that in man in fine structure, except that the former has no smooth muscle fibres as the latter. The nerves in this part are mostly vegetative in nature, while the sensory fibres, very small in number, end in the tunica propria in simple endings, without further entering into the epithelium.

The minute structure of the canine vestibulum nasi is also similar to that in man, except that the former has no pars cutanea with vibrissae, the vestibulum in the front partly going over to the snout, partly to the haired outer skin. The mucosa of the vestibulum is divisible into the corneous part with corneous plate and the non-corneous part, as in the human vestibular mucosa part, but the development of nasal glands is much inferior in the former.

In the submucosa of the vestibulum nasi in dog there are seen many sensory fibres, most of which run toward the corneous mucosa part, to end chiefly with branched endings in the papillae. More intraepithelial fibres are found than in the human counterpart, being in best development at the entrance of the vestibulum. These fibres originate partly in the branched endings in the papillae and partly in single fibres directly penetrating the epithelium. They either end unbranched or branched,

are mostly smooth-surfaced and thin, but more rarely somewhat thicker, and run through or between the epithelial cells, to end sharply or sometimes in nodes in the upper layer of the epithelium.

The snout in dog presumably represents the foremost part of the vestibulum. The epithelium is similar to that of the corneous mucosa part of the vestibulum, being composed of stratified flat epithelium with corneous plate and somewhat greater in height than the latter. The development of papillae is much stronger than in the vestibular corneous part and their arrangement is very regular. No nasal gland is found formed in the tunica propria of the snout.

The snout is far better provided with sensory fibres than the vestibular mucosa part, apparently in proportion to the good development of papillae here. The sensory fibres mostly end in the papillae as branched endings, but in rarer cases, as capsulated end bulbs. Often enough, these endings send out intraepthelial fibres, very large in number, most of which are branched. Some of them are rather thick, though mostly they are minute in size, and generally end sharply or in nodes in the upper layer of the epithelium.

The canine lip, as that in man, is composed of the cutanea part, the transitional part and the mucosa part. The transitional part is subdivided into the outer and inner zones by a shallow furrow. The outer zone occupies only a narrow space. Its epithelium is composed of a stratified flat epithelium with corneous plate and about 3 times thick as the epidermis of the cutanea part. Rather strongly developed papillae are formed beneath the epithelium. Upon going over from the outer to the inner zone, the epithelium suddenly grows in thickness, reaching 4 to 5 times that of the epithelium in the outer zone. No corneous plate is found herein, and villi are formed on the surface. The papillae here are tall and well-developed. The inner zone occupies a far larger area than the outer. Its epithelium diminishes in thickness as the mucosa part is approached, and its height becomes nearly equal to that of the outer zone upon going over to the mucosa part. In the mucosa part, no papillae are formed, unlike that in human lips. Of the mucous membrane of the cheek outside the lip mucosa, only the part corresponding to the level above the rima oris contains papillae.

The sensory supply of the mucous membrane of the lips and cheeks in dog is similar to that in man, the inner zone of the transitional part, the outer zone thereof and the mucosa part being best innervated, in the order named. But the number and the terminal formation of the sensory fibres are much inferior to man, corresponding to the simpler histological picture of the canine mucous membrane. Of the mucosa of the cheek,

only the part corresponding to the level above the rima oris, which is provided with papillae, is rich in sensory fibres.

The sensory terminations in the transitional part of the lip are chiefly represented by simple branched endings, and no such complex endings and terminal bodies as found in man are formed here. Intraepithelial fibres are seen only in the outer zone, as smooth-surfaced fine fibres ending branched or unbranched, with their terminal rami ending sharply or in small nodes.

The sensory fibres in the canine hard palate are much poorer in development than in man, as is the case with cats and hedgehogs. They are, however, greater in number than in cat and their terminations show more complexity. Rather complex branched terminations are found in the papillae, and the number of the intraepithelial fibres is also larger than in the feline couterpart.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Sensory Innervation of the Fore Part of Dorsum Linguae in Hedgehog

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(Received for publication, September 12, 1953)

Many studies have been reported on the nerve supply of the tongue, the recent histological studies on human tongue by Kumagawa,[1] Nakayama,[2] Inazawa[3] and Ogaki,[4] and on dog tongue by Okano,[5] all from this laboratory, deserving special mention. Many noteworthy observations have been published in these reports. We have been granted the opportunity to continue the study on this subject, with hedgehogs as our material, the results whereof we will report in the following.

As for the method of our study, we took tongues of hedgehogs as material, which we fixed for a long time in 10% neutral formol, cut into 40μ frozen sections and stained with Seto's silver impregnation. The large series of beautifully stained preparations obtained thereupon, we subjected to minute microscopic examination, and the observations were reviewed in collation with those on human and some mammalian tongues.

Individual Findings

The sensory nerve fibres in the tongue of hedgehog, as in human (Nakayama)[2] and canine (Okano)[5] tongues, penetrate into the mucous membrane of the tongue, together with vegetative nerve fibres, to distribute in the tunica propria mucosae, in particular, in its papillae. The development of these sensory fibres stands in a close relation with that of the papillae formed subepithelially, as has been established by many investigations conducted in this laboratory.

The papillae in the mucous membrane of the dorsum linguae of hedgehog are incomapratively far weaker than in man and even lower than in dog in development. Moreover, the subepithelial connective tissue is also far worse-developed, so that the lower development of the sensory nerve fibres in hedgehog tongue in comparison with man and dog may be readily deduced from such histological pictures alone.

As for the fine structure of the mucous membrane covering the frontal

part of dorsum linguae in hedgehog, it may be further mentioned that its surface layer is composed of many filiform papillae and a small number of fungiform papillae, the former showing special peculiarity. The epithelium here is composed of a very thick stratified flat epithelium, of a thickness perhaps double that in dog, and with a surface somewhat cornified. The filiform papillae consist of connective tissue papilla stems, penetrating from basal part of the common epithelium into the epithelium at approximately regular intervals. The top of the papilla stems branches out into 2 or 3 secondary papillae. The epithelium continuous with the papilla stem is composed of elongated epithelial cells stretching vertically beyond the surface of the common epithelium to form a epithelial villus. These villi jut out into the oral cavity at fairly regular intervals and frequently end in 2 or 3 sharp branches respectively. These villious cells are provided with a cell nucleus and are probably corneous-plated in nature.

As described above, the formation of the filiform papillae in hedgehog tongue is much different from those in man and dog. But after all, since the development of the connective tissue papilla stem which is related with sensory innervation is weaker than that in man and dog, it follows that the sensory nerve fibres in this part are also worse-developed in hedgehog.

Unlike the filiform papillae, the fungiform papillae in hedgehog tongue are essentially not different from those in man and dog. Their papilla stem projects strongly into the epithelium, forms a few very minute secondary papillae and is covered by an extremely thin stratified flat epithelium. The surface of the epithelium forms a thin corneous plate composed of nucleated flat cells. Frequently, taste-buds are formed in the epithelium facing the oral cavity, quite analogous to the human and the canine counterparts. Seeing that the fungiform papillae are similar to those in dog in form and size, it may be easily understood that the development of sensory fibres in these papillae in hedgehog is not much different from the same in dog.

As may be well surmised from the above described histological picture, the nerve plexus found in the ill-developed subepithelial connective tissue on the dorsum linguae in hedgehog is also very poorly formed, hardly any nerve plexus worthy of the name being observable. Only directly under the fungiform papillae, true nerve plexus is found formed of fairly thick nerve bundles.

The sensory nerve fibres supplying the filiform papillae of hedgehog are represented by comparatively thick medullated fibres, with their terminations in their papilla stem, but as the total number of the sensory fibres is very limited, many of the filiform papillae lack any sensory fibre altogether. The terminations are of simpler formation than in those in

Fig. 1

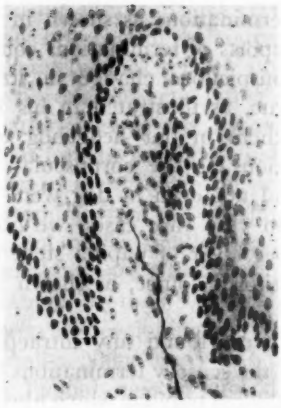

Fig. 2

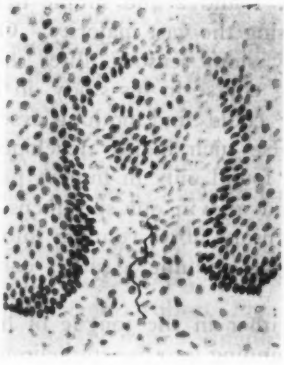

Fig. 1. An unbranched sensory termination ending sharply in the papilla stem of a filiform papilla in the anterior part of hedgehog tongue. Seto's impregnation. X 400, reduced to 2/3.

Fig. 2. Ditto.

Fig. 3

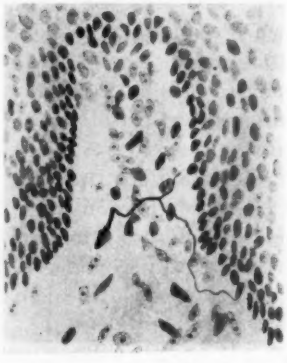

Fig. 4

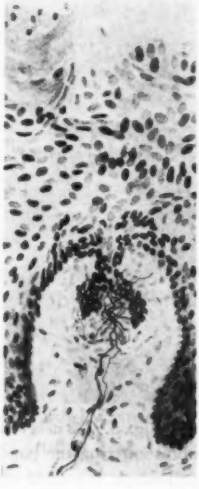

Fig. 3. A bifurcated sensory termination ending sharply in the papilla stem of a filiform papilla in the anterior part of hedgehog tongue. Same staining, X 600, reduced to 2/3.

Fig. 4. Somewhat complex loop-like sensory termination originated in 2–3 sensory fibres in the papilla stem of a filiform papilla in the anterior part of hedgehog tongue. Same staining. X 300, reduced to $\frac{1}{2}$.

dog (Okano[5]), such sharply ending unbranched terminations as shown in Figs. 1 and 2 and simple branched terminations as shown in Fig. 3 comprising the vast majority. Okano[5] reports of terminations composed by very stout sensory fibres showing conspicuous change in size and winding course in his study on dog tongue, as illustrated in his Fig. 5, but we could find nothing resembling them in our hedgehog tongue. Of course such highly developed formations as the corpuscular terminations found only in human papillae and not even in dog were not to be found also in our specimens. Only in very rare cases, we observed some sensory fibres running into the upper layer of papilla stem to end there after running a rather complex ansiform course, as shown in Fig. 4.

Neither in the tongue of hedgehog were found any intraepithelial fibres running into the epithelium through the sensory terminations formed in the filiform papillae, alike the canine filiform papillae which also lacked such intraepithelial fibres, a fact probably due to the cornified nature of the epithelium.

In the fungiform papillae, in hedgehog as well as in dog, fairly remarka-

Fig. 5

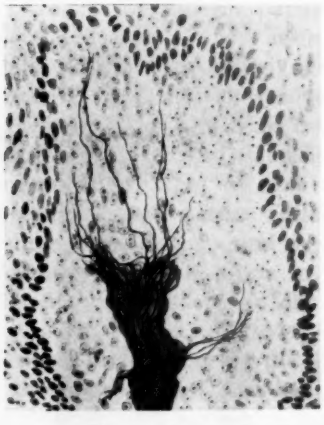

Fig. 6

Fig. 5. A nerve bundle composed of thin vegetative and thick sensory fibres entering the papilla stem of a fungiform papilla and some unbranched and simple branched sensory terminations originated in the former and ending sharply in the subepithelial layer. Anterior part of hedgehog tongue. Same staining. X 400, reduced to ½.

Fig. 6. Unbranched and branched sensory terminations formed in the papilla stem of a fungiform papilla and some extragemmal sensory fibres. Anterior part of hedgehog tongue. t taste-bud. Same staining. X 400, reduced to ½.

ble nerve plexus is found formed at their basis. The nerve bundles originated in the plexus ascend vertically through the papilla stem and divide into finer branches in the course. The nerve elements consist of numerous comparatively thick sensory fibres and some thin vegetative fibres. The former fibres mostly end either sharply in unbranched or simple branched terminations, few if any forming corpuscular terminations like those observed in human fungiform papillae (Figs. 5 and 6).

Also in these fungiform papillae there are found some intraepithelial fibres running from the subepithelial tissue into the epithelium facing the cavity, but in far smaller number than in dog. The sensory fibres for the taste-buds are also very poorly developed, forming merely simple terminations at their basis. In some rare cases, however, these fibres are observed developing into intra- or extragemmal fibres (Fig. 6).

Finally, Nakayama[3] has proved existence of muscle spindles in the human lingual muscles and detailed the innervation of these spindles, but all our strenous search for such formation in our hedgehog proved futile, as Okano[5] also failed to find such spindles in his canine tongue.

Summary

Corresponding to the development of the subepithelial connective tissue and the papilla stems of the filiform papillae in the fore half of the dorsum linguae of hedgehog, far lower than that in man and even poorer than that in dog, the sensory fibres distributed in this part are further backward in development in hedgehog than in dog, many of the filiform papillae lacking sensory fibres altogether. Even in the cases when sensory fibres enter the filiform papillae, their terminal formations are always extremely simple, generally ending in sharp unbranched or simple branched terminations, except in very rare cases where ansiform terminations are found. No intraepithelial fibres are found here.

The fungiform papillae in hedgehog are similar to those in dog in their formation, and the development of sensory fibres penetrating into them is also much in the same stage. The rather numerous sensory fibres here also end in most cases sharply in unbranched or in simple branched endings. Intraepithelial fibres are rarely proved in the epithelium of fungiform papillae facing the oral cavity. The development of sensory fibres related with taste-buds is also very poor, but a few intra- or extragemmal fibres have been found.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Biochemical Studies on Carbohydrates

CLXVII, Structural Study of the Group Carbohydrate from Pig Stomach Mucus

Ninth Paper*

By

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(Received for publication, September 13, 1953)

The present account concerns chiefly L-fucose in the group carbohydrate from pig stomach mucus. The presence of L-fucose in this polypeptide-polysaccharide complex was mentioned in the addendum to the 7th Paper.[1]

A specimen of the group substance prepared by the procedure hitherto employed was purified by fractionation with alcohol. The 50% alcohol-soluble part (Ch. I) of molecular weight 3650 (calc. from the iodine value) contained 9.5% L-fucose namely 2 molecules per molecule and 42.5% glucosamine namely 9 molecules. By heating with 1 *N* acetic acid at 100°C for 1 and 3 hours, it gave scission products which will be referred to as Ch. II and Ch. III respectively. The former product contained 5.1% L-fucose and 43.4% glucosamine. Since its molecular weight was calculated at 3470, the content of L-fucose corresponded to 1 molecule per molecule and that of glucosamine to 9 molecules. In the latter product whose molecular weight was 2940, L-fucose amounted only to 0.8% and glucosamine to 44.1%, suggesting that nearly 2 molecules of L-fucose and 1 molecule of glucosamine in Ch. I had been cleft off by the 3 hours' treatment with acetic acid. Galactose content increased thereby in inverse proportion to molecular weight.

The Chs. I, II and III all gave the positive indirect Osaki-Turumi reaction[3] distinctly. Therefore, it is obvious that, in any of them, the reducing end of the polysaccharide chain must have been occupied by an acetylglucosamine molecule. Furthermore, from the compositions of those preparations it is assumed that an L-fucose molecule stood at the non-

* Read before the 25th General Meeting of the Japanese Biochemical Society, Tokyo, April 26–28, 1953, and the preliminary note has been published in this Journal[2].

reducing end of Ch. I, an acetylglucosamine molecule and the remaining L-fucose molecule neighbouring in turn.

Next, Ch. I was examined as to its consumption of periodic acid under the conditions previously described[4]. It reduced 3.1×10^{-3} mole of the oxidant per g., that is, 11.3 moles per mole, nearly coinciding with the result obtained on a former preparation of the group substance in the 4th Paper[5] (Ch. I here resembled that former preparation regarding molecular weight too). Chs. I, II and III were then subjected to oxidation with sodium metaperiodate at pH 5.0 according to Aminoff & Morgan[6] and Jeanloz[7]. They used 3.3×10^{-3}, 3.1×10^{-3} and 3.3×10^{-3} mole of the oxidant per g. (12.0, 10.8 and 9.7 moles per mole) respectively. Analysis of Ch. I and its oxidation products revealed that all the acetylglucosamine molecules exclusive of the one at the reducing terminal of the chain hade not been destroyed by the metaperiodate. As will be discussed below, the intermediate non-amino sugars reduced 1 molecule of the agent per molecule and the terminal 2 molecules, in accord with what had been found in the foregoing study. It might be thus plausible that the L-fucose molecules in the pyranose form are bound with the neighbouring acetylglucosamine molecules at just the same positions as the galactose molecules in the chain. Whether they are the α- or β-isomer will be contemplated elsewhere.

Ch. I was Group A as well as Group O (and E) potent. By treatment of it with hot acetic acid, the both activities were diminished. Although Chs. II and III were found equally Group A potent, the latter in which only a minute amount of L-fucose remained uncleft was much less Group O (and E) active than the former. Hence, L-fucose seems to be an intrinsic component for the Group O (and E) activity but non-intrinsic for Group A activity. To add, the products after $NaIO_4$-oxidation possessed none of the serological properties including the anti-sheep hemolytic.

Experimental

Preparation of Chs. I–III

Ch. I. A specimen, prepared from pig stomach mucus as already reported, contained 4.0% N, 42.7% glucosamine (Blix[8]), 34.7% galactose (Masamune & Ogawa[9]), 14.0% L-fucose (Masamune & Ogawa[10]) and 1.2 % ash (Pregl without use of H_2SO_4). 6 g. of it were dissolved in 150 cc. of water (A small part insoluble was discarded) and electrodialyzed until no more change of pH occurred. It was then distilled in vacuo to about 30 cc., an equal volume of abs. alcohol added and the precipitate centrifuged off. To the opalescent supernatant were added 300 cc. of abs. alcohol containing a little NaCl. The flocculent precipitate here was washed with abs. alcohol, which contained NaCl as above, and dried in a

vacuum desiccator over H_2SO_4. Crop, 5.4 g. Dissolution in water, electrodialysis and fractionation with alcohol was processed over again, and 5.0 g. of a white powder were given—Ch. I. Biuret negative. Molisch and the indirect Osaki-Turumi intensively positive.

Ch. II. 0.5 g. of Ch. I was refluxed with 10 cc. of 1 *N* acetic acid in a boiling water-bath for 1 hour. When cold, the hydrolysate was diluted to 40 cc. and dialyzed in a cellophane bag against running water until the solution became neutral, followed by vacuum distillation to about 15 cc. To the condensate were added 10 volumes of an alcohol-ether (1 : 1 by volume) mixture, then the deposit in flakes was centrifuged, washed with the alcohol-ether mixture and dried in vacuo over H_2SO_4. A grey powder obtained weighed 0.34 g. Biuret faintly positive; Molisch and the indirect Osaki-Turumi strongly positive.

Ch. III. 0.5 g. of Ch. I was treated with acetic acid similar to above by heating for 3 hours. 0.3 g. was the yield. The qualitative tests resulted same as in Ch. II.

Chemical and Physical Properties of the Preparations above

Paper partition chromatography of the sugar components. Masamune & Yosizawa technique[11] was applied. Hydrolysis of Chs. I–III was effected by heating 20 mg. each of them with 2 cc. of 1 *N* H_2SO_4 for 5 hours, and after removal of most of sulfuric acid with baryta, the hydrolysates were distilled under reduced pressure to about 0.2 cc. (pH of the condensates 5.8), whose 0.01 cc. portions were sampled. The standard solution was 2% with respect to each individual sugar and 0.015 cc. was sampled. As Fig. 1 shows, all the carbohydrate preparations were found to contain glucosamine, galactose and L-fucose (The L-fucose spot was very weak in Ch. III) but no sugar else.

Quantitative analysis and optical measurement. The results are embodied in Table I.

Group Specificity and Antilytic Activity of the Carbohydrate Preparations[11a]

Group activities (A, B, O and E) were determined as already described by Masamune *et al.*[12]

Group A specificity. The human B serum had titre 160 and was diluted 20-fold. Chs. I, II and III inhibited the agglutination of A erythrocytes at dilutions of $1 : 6.4 \times 10^5$, $1 : 4 \times 10^4$ and $1 : 4 \times 10^4$ respectively (Table II).

Group B specificity. The human A serum had titre 160 and was diluted 20-fold. Chs. I–III were all devoid of this potency (Table II).

Group O specificity. A normal anti-O goat serum, which had titre 320 against human O erythrocytes (The agglutinin proved to be absorbable

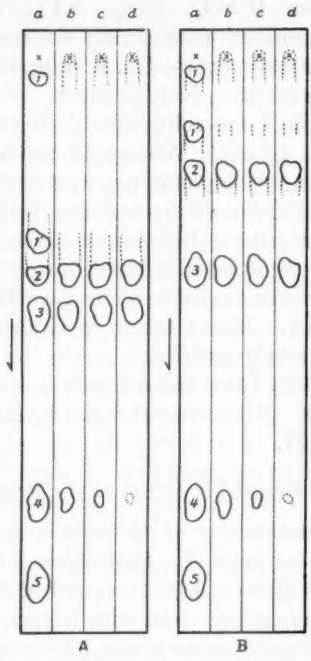

Fig. 1. Chromatograms developed on slivers 60 cm. × 5 cm. in the descending way with (A) butanol-pyridine-water (5 : 3 : 2) and (B) *n*-butyl-acetate-acetic acid-ethanol-water (3 : 2 : 1 : 1) of the hydrolysates of Chs. I, II and III. Temperature of test 23–26°C. Duration of test 24 hrs. Indicator aniline-hydrogen phthalate.

a : reference run, b : Ch. I, c : Ch. II, d : Ch. III, 1 and 1′ : chondrosamine sulfate, 2 : glucosamine sulfate, 3 : galactose, 4 : L-fucose, 5 : rhamnose.

with the saliva of O secretors, but not with that of O non-secretors) was diluted 80-fold. Chs. I, II and III inhibited the agglutination of O erythrocytes at dilutions of $1 : 6.4 \times 10^4$, $1 : 8 \times 10^3$ and $1 : 10^3$ respectively (Table III).

E specificity. An eel serum having titre 800 against human E erythrocytes and titre 40 against human e erythrocytes was diluted 200-fold. Chs. I, II and III inhibited the agglutination of O erythrocytes by the diluted eel serum at dilutions of $1 : 3.2 \times 10^4$, $1 : 4 \times 10^3$ and $1 : 10^3$ (Table III).

Anti-sheep-hemolysin activity

Masamune *et al.*[12] were followed by using an immune anti-A rabbit serum diluted to titre 4. The results (Table IV) revealed that the carbo-

TABLE I

Analytical Figures, Molecular Weights and Optical Rotations of Chs. I–III

Analysis and else	Ch. I	Ch. II	Ch. III	Ch. I	Ch. II	Ch. III
Composition						
	In per cent			In equivalents per equivalent weight		
N*	4.1	4.6	4.7	1.23	1.36	1.36
Glucosamine†	42.5	43.4	44.1	1.0	1.0	1.0
Galactose‡	34.3	35.5	38.6	0.80	0.81	0.87
L-Fucose§	9.5	5.1	0.8	0.24	0.13	0.02
Acetyl//	10.7	10.4	10.6	1.05	1.00	1.00
Ash¶	3.0	1.7	1.2			
Iodine value and molecular weight calc. therefrom						
Iodine value**	5.8	6.1	7.2			
Mol. wt.	3650	3470	2940			
Optical rotation						
$[\alpha]_D$	$\frac{-0.59\times100}{1\times3.88}=-15.2°$ (in H_2O, 7°C); $\frac{-0.34\times100}{1\times1.25}=-27.2°$ (in 0.01 *N* NaOH, 8°C)	$\frac{-0.19\times100}{1\times1.33}=-14.3°$ (in H_2O, 16°C)				

* Micro Kjeldahl. † Blix method. ‡ Masamune & Ogawa method[9], § Masamune & Ogawa method[10]. // Suzuki's modification of Friedrich, Rapoport & Sternberg method. ¶ Pregl method without use of H_2SO_4. ** Number of cc. of 0.1 *N* iodine solution reduced by 1 g. of a substance.

TABLE II

Agglutination of Human A and B Erythrocytes by Normal Antisera treated with Ch. I, II or III

− : no agglutination, ± : faint aggl., + : slight aggl., ++ : moderate aggl., +++ : marked aggl.

Ch.	Group of red cells	Dilution of Chs. (1 :)													
		10^2	2×10^2	4×10^2	8×10^2	10^4	2×10^4	4×10^4	8×10^4	1.6×10^5	3.2×10^5	6.4×10^5	1.28×10^6	2.56×10^6	∞
I	A					−	−	−	−	−	−	−	±	+	++
	B	++	++	++	++										
II	A					−	−	−	±	+	+	+	++	++	
	B	++	++	++	++										
III	A					−	−	−	+	+	+	+	++	++	
	B	++	++	++	++										

hydrate preparations were of identical potency, inhibiting hemolysis at a dilution of $1:1.6\times10^5$.

TABLE III

Agglutination of O Erythrocytes by a Normal Anti-O Goat Serum or an Eel Serum treated with Ch. I, II or III

−: no agglutination, ±: faint aggl., +: slight aggl.

Ch.	Antiserum	Dilution of Chs. (1:)									
		10^3	2×10^3	4×10^3	8×10^3	1.6×10^4	3.2×10^4	6.4×10^4	1.28×10^5	2.56×10^5	∞
I	Anti-O goat serum	−	−	−	−	−	−	−	±	+	+
	Eel serum	−	−	−	−	−	−	±	+	+	+
II	Anti-O goat serum	−	−	−	−	+	+	+	+	+	
	Eel serum	−	−	−	±	+	+	+	+	+	
III	Anti-O goat serum	−	±	+	+	+	+	+	+	+	
	Eel serum	−	+	+	+	+	+	+	+	+	

TABLE IV

Hemolysis by an Anti-A Rabbit Serum, treated with Ch. I, II or III, and a Complement

−: no hemolysis, ±: faint hemol., +: slight hemol., ++: marked hemol., +++: nearly complete hemol., ++++: complete hemol.

Ch.	Dilution of Chs. (1:)								
	4×10^4	8×10^4	1.6×10^5	3.2×10^5	6.4×10^5	1.28×10^6	2.56×10^6	5.12×10^6	Control*
I	−	−	−	±	+	++	+++	++++	++++
II	−	−	−	+	+	++	++	++++	
III	−	−	−	±	+	++	+++	++++	

* Without the complement and substrate.

Oxidation with Periodic Acid or Sodium Metaperiodate of the Carbohydrate Preparations

Oxidation with periodic acid of Ch. I. It was processed by the procedure Yosizawa[4] had described:— To 1 cc. of a 1.2% solution of the carbohydrate preparation was added 0.2 cc. of 0.4135 *M* periodic acid, and the mixture was stood at 20°C for 70 minutes, when the change of rotation ceased. The blank run was carried out in parallel. 0.0455×10^{-3} mole of the oxidant remained unreduced. The consumption comes to $\frac{(0.0827-0.0455)\times10^{-3}}{12}\times10^3=3.1\times10^{-3}$ mole per g. and 11.3 moles per mole of the carbohydrate whose molecular weight was 3,650 (Cf. Table I).

Oxidation with sodium metaperiodate of Chs. I–III at pH 5.0. Aminoff & Morgan and Jeanloz were followed. 30 mg. of a carbohydrate preparation were taken up in 5 cc. of 0.1 *M* acetate buffer at pH 5.0 and oxidized with 1 cc. of 0.3024 *M* $NaIO_4$ at room temperature (16–18°C). 0.5 cc. portions were pipetted out at intervals from the reaction mixture, and after addition of 2 cc. of 9% (w/v) $NaHCO_3$, 1 cc. of 0.1 *N* arsenious oxide and 0.1 cc. of 20% (w/v) KI, stood for 15 minutes at room temperature to titrate with 0.02 *N* (or 0.01 *N*) iodine solution (indicator 1% starch solution). Chs. I, II and III used 3.3×10^{-3}, 3.1×10^{-3} and 3.3×10^{-3} mole of $NaIO_4$ per g., i.e., 12.0, 10.8 and 9.7 moles per mole. The results are illustrated in Fig. 2 (Regarding molecular weights of Chs. II and III, see Table I).

As a control, glucosamine hydrochloride, N-acetylglucosamine and α-methyl-N-acetylglucosaminide were oxidized in a similar manner, taking 5 cc. each of 0.12% solutions of the first two sugars and a 0.24% solution of the last sugar in the acetate buffer solution. Temperature of test 14—16°C. 5.0, 4.7 and 1.0 moles of sodium metaperiodate were reduced respectively by the sugars per mole in accord with Jeanloz's findings (Fig. 3).

Fig. 2

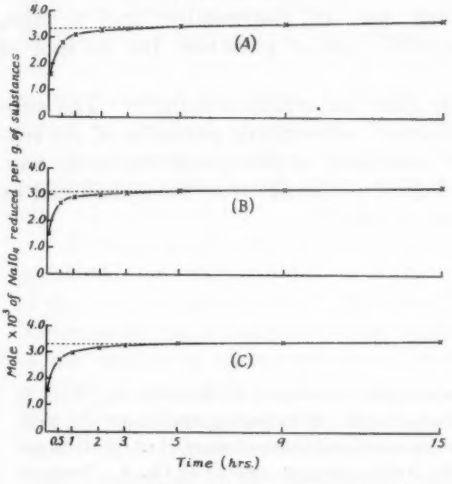

Fig. 3

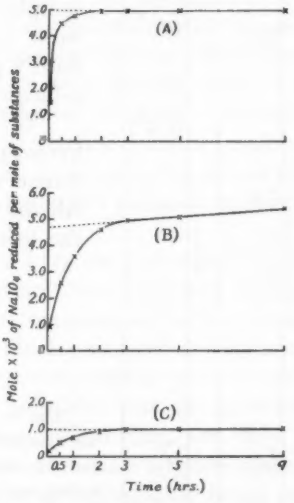

Fig. 2. $NaIO_4$ consumptions by Chs. I (A), II (B) and III (C) at pH 5.0 and 16–18°C as a function of time.

Fig. 3. $NaIO_4$ consumptions by glucosamine hydrochloride (A), N-acetylglucosamine (B) and α-methyl-N-acetylglucosaminide (C) at pH 5.0 and 14–16°C as a function of time.

On the Product of Ch. I after Oxidation with Sodium Metaperiodate

Separation procedure. 300 mg. of Ch. I were dissolved in 50 cc. of 0.1 *M* acetate buffer at pH 5.0 and after addition of 10 cc. of 0.3024 *M* $NaIO_4$, stood at room temperature (16–18°C) for 3 hours. To decompose the excess periodate, the mixture was further stood for 1 hour at room temperature together with 2 cc. of ethylenglycol. It was then filled up with water to 300 cc., dialyzed until it turned almost neutral and distilled in vacuo to about 10 cc. (During the condensation thin films precipitated, which were not removed), followed by addition of 100 cc. of abs. alcohol. The deposit was centrifuged, washed with the alcohol and dried in a vacuum-desiccator over H_2SO_4. 240 mg. of a white powder were yielded. Molisch faintly positive; the indirect Osaki-Turumi weakly positive; Schiff test for aldehyde remarkably positive. N 4.3%, glucosamine (Blix) 41.0%, acetyl 9.8%, (equivalent ratios 1.34 : 1.0 : 0.99); ash 1.6%.

Paper partition chromatography of the sugar components. Masamune and Yosizawa procedure was employed. 20 mg. of the substance were subjected to hydrolysis (100°C, 5 hrs.) with 2 cc. of 1 *N* H_2SO_4 and the hydrolysate removed from most H_2SO_4 was condensed to about 0.15 cc., of which 0.008 cc. portions were sampled. The chromatograms reproduced in Fig. 4 show a strong spot of glucosamine and a faint, hardly perceptible spot of galactose, but no spot of L-fucose.

Group specificity and antilytic activity.[11a] The antihemoagglutinative and antilytic potencies of the substance were examined as above with the same sera. All the serological properties were seen abolished by the oxidation (Table V).

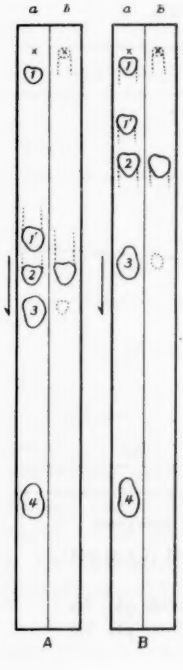

Fig. 4. Chromatograms developed on slivers 60 cm. × 5 cm. in the descending manner with (A) butanol-pyridine-water (5 : 3 : 2) and (B) *n*-butylacetate-acetic acid-ethanol-water (3 : 2 : 1 : 1) of the hydrolysate of the $NaIO_4$-oxidation product of Ch. I. Temperature of test 24–26°C. Duration of test 24 hrs. Indicator aniline-hydrogen phthalate.

a : reference run, b : the oxidation product of Ch. I, 1 and 1′ : chondrosamine sulfate, 2 : glucosamine sulfate, 3 : galactose, 4 : L-fucose.

TABLE V

Antihemoagglutinative and Antihemolytic Potencies of the $NaIO_4$-oxidation Product of Ch. I

±: faint agglutination or lysis, +: slight aggl. or lysis, ++: marked aggl. or lysis, +++: extreme aggl. or complete lysis.

Antiserum	Dilution of the oxidation product (1:)							
	10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	∞	Control*
Normal B serum	++	++	++	++	++	++	++	
Normal A serum	++	++	++	++	++	++	++	
Anti-O goat serum	±	+	+	+	+	+	+	
Eel serum	+	+	+	+	+	+	+	
Anti-A rabbit serum (+complement)	±	+	+	++	+++	+++		+++

* Without the substance and complement.

DISCUSSION

From its molecular weight and analyses, Ch. I is considered to contain 7 molecules of galactose besides 9 molecules of acetylglucosamine and 2 molecules of L-fucose. And as described above, an acetylglucosamine molecule stands at the reducing terminal of the polysaccharide chain as the first hexose unit and an L-fucose molecule, an acetylglucosamine molecule and the other L-fucose molecule represent the three hexose units in the order arranged beginning with the non-reducing terminal. Hence, it might be justified to replace two acetylglucosamine-fucoside molecules for the last two acetylglucosamine-galactoside molecules (numbered from the reducing disaccharide unit) in the former formula assigned to the present carbohydrate.[13]

Next, as to the periodic acid use of Ch. I by the procedure hitherto employed, the intermediate non-amino sugar molecules reduced 1 molecule of the oxidant per molecule, because, if the consumption by the terminal hexose units (the L-fucose molecules in the chain are assumed as a pyranose) of the oxidant is subtracted from the total consumption, the balance in number of molecules (11.3−3=8.3) nearly coincides with the number of the intermediate non-amino sugar molecules. Therefore, not only the intermediate galactose molecules but also the intermediate L-fucose molecule used 1 molecule of the oxidant per molecule, most probably being combined in similar modes with the neighbouring acetylglucosamine molecules, in other words, C_I of that L-fucose molecule is linked with C_{IV} of the preceding acetylglucosamine molecule and C_{II} of it with C_I of the succeeding acetylglucosamine molecule. An L-fucose molecule bound at its

C_{IV} in place of C_{II} besides at C_I also reduces one molecule of $NaIO_4$ per molecule, but the union at C_{II} appears more probable (Cf. Masamune *et al.*[14]).

By experimenting with acetylglucosamine and α-methyl-N-acetylglucosaminide by the procedure of Aminoff & Morgan and Jeanloz, it was substantiated that 0.7 molecule of $NaIO_4$ is used by the first two C atoms of acetylglucosamine free or bound at one of the atoms C_{III}–C_{VI} as the authors reported (Comp. Nicolet & Shinn[15] and Neuberger[16]). So the theoretical $NaIO_4$ uses by the Chs. I–III per molecule become 11.7, 10.7 and 9.7 molecules:

Ch. I $A-(G-A)_7-F-A-F$
$1.7+(1+0)\times 7+1+0+2=11.7$
Ch. II $A-(G-A)_7-F-A$
$1.7+(1+0)\times 7+1+1=10.7$
Ch. III $A-(G-A)_6-G-A$
$1.7+(1+0)\times 6+1+1=9.7$
(A=acetylglucosamine, G=galactose, F=L-fucose)

The actual values 12.0, 10.8 and 9.7 molecules closely coincide with the calculated.

The L-fucose molecules were assumed above to be a pyranose. If they were a furanose, the intermediate joined at its C_V with the succeeding acetylglucosamine would also reduce 1 molecule of the oxidant but the terminal no more than 1 molecule, entailing the decrease of the total amount used by the polysaccharide.

Summary

1. The structure of the group carbohydrate from pig stomach mucus (prepared as already reported and purified) (Ch. I) was investigated particularly in connection with L-fucose molecules in it.

2. The group substance Ch. I was heated with acetic acid. And the material and hydrolysis products (Chs. II and III) were oxidized with periodic acid and with sodium metaperiodate. The consumption of HIO_4 by Ch. I and those of $NaIO_4$ by Chs. I, II and III and the chemical properties of Chs. I–III and of the $NaIO_4$-oxidation product of Ch. I suggested that the original group substance (Ch. I) contains two L-fucose molecules in place of two galactose molecules as the last and last but two hexose units in the formula already put forward[13] for it and further that the L-fucose molecules are bound with neighbouring acetylglucosamine molecules similar to the corresponding galactose molecules in the same formula.

3. Serological properties of the original substance and its hydrolysis and oxidation products were assayed and the meaning of L-fucose in the

serological aspect was discussed (See the introductory note).

The expenses of this work were defrayed by a grant from the Education Department given through the Grant Committee for Scientific Researches.

H. Masamune

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Biochemical Studies on Carbohydrates

CLXVIII. Fractions of Pig Gastric Mucus and the Carbohydrate Preparations therefrom*

By

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(Received for publication, September 13, 1953)

Pig gastric mucus was fractioned into mucus-mucin (Sinokawa[1]) and three other fractions. Those fractions comprised hexosamine (chondrosamine and glucosamine), galactose, L-fucose and mannose in approximately same molecular proportions (1 : 0.70–0.76 : 0.31–0.46 : 0.03–0.05), suggesting that they involved an identical polysaccharide or identical polysaccharides in equal proportions. Next, three of the fractions including the mucus-mucin were subjected to alkaline cleavage at 20°C (A fourth amounted too small for the purpose), and the mucus-mucin also to alkaline cleavage at 0–3°C and 30–35°C and to tryptic digestion, followed by separation of the group carbohydrate (containing a small amino acid-grouping). The substance obtained by scission of the protein with alkali at the lowest temperature and that by scission by the aid of trypsin were composed of all the sugar components above exclusive of mannose and as Group A potent as the most active mucus-fraction, but the ones prepared by the treatment at higher temperatures were composed of only glucosamine, galactose and L-fucose, being devoid of chondrosamine, and much less Group A potent. E activity was not remarkably damaged even by alkali at 30–35°C. Judging from the analytical figures of those carbohydrate preparations, first, one and the same group carbohydrate appears to constitute the mucus fractions, and second, in the fully active form of the group carbohydrate, not only the whole acetylchondrosamine molecules but also a part of galactose and L-fucose are linked by an alkali-labile union or alkali-labile unions with the alkali-stable portion made up of acetylglucosamine, galactose and L-fucose.

* Read before the 25th General Meeting of the Japanese Biochemical Society, Tokyo, April 26–28, 1953. A preliminary note has been published in this Journal.[1a]

Experimental

Fractionation of Pig Stomach Mucus

A batch of the mucus was collected from pig stomachs. 100 g. of it were shaken with 500 cc. of 1% sodium bicarbonate and 30 cc. of toluene for 2 days and centrifuged. Swollen mucin formed the upper layer which was put away. The lower watery layer (turbid) was shaken with changes of toluene as above to separate the remaining mucin in solution. The final centrifuged lower layer was set aside for the fractionation below. The mucin portions given were combined and thoroughly washed with 1% sodium bicarbonate in a separatory funnel. They were then suspended in 200 cc. of water, acidified to pH 2.0 with dilute HCl and shaken well again. The mucin collecting at the top of the fluid on standing was further subjected to washing with very dilute HCl several times, subsequently treated with alcohol and ether and finally dried to a constant weight in vacuo over H_2SO_4. 2.1 g. of a grey powder were produced—**Fr. I** (mucus-mucin of Sinokawa). Phosphorus was detected but in trace. Next, the fluid freed from the mucus-mucin and the alkaline washings were united (7 *l.* in all), adjusted to pH 8.0 with dilute HCl and divided into 1 *l.* portions. The portions were separately shaken with a mixture of 15 cc. of toluene and 30 cc. of chloroform for 2 days and centrifuged. In each run the watery upper layer was decanted out, and the gel was shrunk by the aid of 300 cc. of alcohol, washed with two 20 cc. alcohol portions and dried as above. The grey powders given which amounted to 0.4 g. in total were put together—**Fr. II.** It contained a small amount of phosphorus. The opalescent fluids set apart from the gel were also treated separately. Each was acidified to pH 2.0 with dilute HCl and the occurring precipitate was centrifuged, washed with alcohol and dried. Grey powders here obtained and combined weighed 0.2 g. altogether—**Fr. III.** P content very small. The centrifuged acid supernatant (opalescent) were dialyzed against running water until they turned neutral, united and after distillation in vacuo to about 40 cc., precipitated with 250 cc. of alcohol. The deposit was washed with alcohol and dried similar to above—**Fr. IV** (a grey powder). Crop, 1.6 g. P was detected distinctly.

All the fractions gave the positive biuret-, Molisch- and indirect-Osaki-Turumi-reaction.

Solubility and Chemical and Serological Properties of the Fractions

Solubility. Frs. I–II were insoluble in water, but dissolved in 0.01 *N* NaOH in part. The remaining fractions (III–IV) were soluble in water

as well as in the dilute alkali.

Paper chromatography of the sugar components. Of each of the fractions, 50 mg. were heated with 5 cc. of 1 *N* H_2SO_4 in a sealed tube at 100°C for 5 hours. The hydrolysate was nearly neutralized with baryta and centrifuged. The supernatant and washing were distilled together in vacuo to about 0.25 cc., adjusted to pH 5.8 with dilute H_2SO_4 and 0.018 cc. and 0.02 cc. portions respectively were sampled in the cases of Frs. I and II and in those of the remaining fractions to chromatograph after Masamune & Yosizawa[2]. The standard solution for reference runs was 2% with respect to each individual sugar and 0.015 cc. portions were sampled. As Fig. 1 shows, all the mucus fractions gave distinct spots at the positions

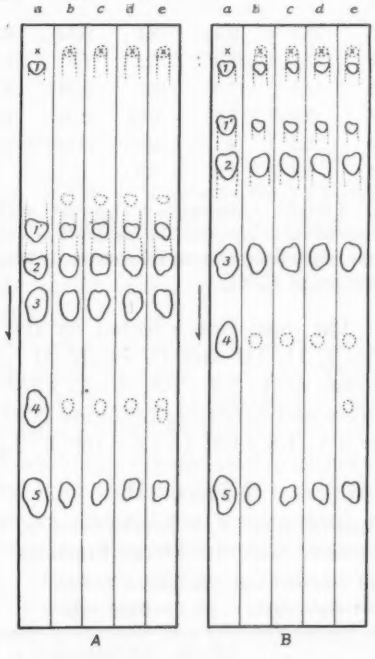

Fig. 1. Diagrammatical reproductions of the sugar chromatograms of the mucus fractions developed with (A) butanol-pyridine-water (5 : 3 : 2) and (B) *n*-butylacetate-acetic acid-ethanol-water (3 : 2 : 1 : 1). Temperature of test 23–26°C. Duration of test 24 hrs. Indicator aniline hydrogen phthalate.
a : reference run. b : Fr. I, c : Fr. II, d : Fr. III, e : Fr. IV.
1 and 1′ : chondrosamine sulfate, 2 : glucosamine sulfate, 3 : galactose, 4 : mannose, 5 : L-fucose.

corresponding to chondrosamine, glucosamine, galactose and L-fucose and

a very weak spot at the position corresponding to mannose. Besides, a faint spot was detected in the case of Fr. IV.

Analysis. The fractions gave figures in Table I. Most probably they contained sugars hexosamines, galactose, L-fucose and mannose in the same molecular proportions of approximately 1.0 : 0.73 : 0.39 : 0.05.

TABLE I

Composition of the Fractions of Pig Stomach Mucus

Analysis	In per cent				In equivalents per equivalent weight			
	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. I	Fr. II	Fr. III	Fr. IV
N*	10.7	11.9	9.4	9.4	9.70	8.90	5.02	5.64
Hexosamine†	14.1	17.1	23.9	21.3	1.0	1.0	1.0	1.0
Galactose‡	10.5	12.1	17.2	16.2	0.74	0.70	0.72	0.76
Mannose‡	0.7	0.9	1.1	0.6§	0.05	0.05	0.05	0.03
L-Fucose//	6.0	5.5	6.7	6.2	0.46	0.35	0.31	0.32
Ash¶	0.4	1.9	1.8	5.3				

* Micro Kjeldahl. † Blix.[3] ‡ Masamune & Ogawa.[4] § The total E at 495 mμ minus the partial corresponding to galactose was employed as such for calculating out mannose because of the neglectable amount of pentose. // Masamune & Ogawa.[5] ¶ Pregl method without use of H_2SO_4.

Group specificity. The assay was effected by the methods[6] hitherto employed by us. Frs. I, II, III and IV inhibited isoagglutination of A erythrocytes at dilutions of $1 : 3.2\times10^6$, $1 : 3.2\times10^6$, $1 : 2.56\times10^7$ and $1 : 1.28\times10^7$ respectively and heteroagglutination of O erythrocytes by an eel serum at dilutions of $1 : 1.6\times10^5$, $1 : 1.6\times10^5$, $1 : 3.2\times10^5$ and $1 : 6.4\times$

TABLE II

Occurrence of Agglutination of A Erythrocytes by Normal Serum, treated with the Mucus Fractions

The B serum had titre 160 and was diluted 20-fold. − : no agglutination, ± : faint aggl., + : slight aggl., ++ : marked aggl.

Mucus fraction	Dilution of substances (1 :)					
	3.2×10^6	6.4×10^6	1.28×10^7	2.56×10^7	5.12×10^7	∞
Fr. I*	−	±	+	+	++	++
Fr. II*	−	±	+	+	++	
Fr. III†	−	−	−	−	+	
Fr. IV†	−	−	−	+	+	

* The substance was swollen in 0.01 *N* NaOH containing 0.86% NaCl as an original suspension. † Dissolved in 0.01 *N* NaOH containing 0.86% saline.

10^5 respectively. However, they proved to be Group B inactive even at a dilution of 1 : 100 (An A serum of titre 160 was diluted 20-fold). See Tables II–III.

TABLE III

Occurrence of Agglutination of O Erythrocytes by an Eel Serum treated with the Mucus Fractions

The eel serum employed had titre 800 against O erythrocytes and was diluted 200-fold. − : no agglutination, ± : faint aggl., + : slight aggl.

Mucus fraction	Dilution of substances (1 :)						
	4×10^4	8×10^4	1.6×10^5	3.2×10^5	6.4×10^5	1.28×10^6	∞
Fr. I*	−	−	−	+	+	+	+
Fr. II*	−	−	−	+	+	+	
Fr. III†	−	−	−	−	+	+	
Fr. IV†	−	−	−	−	−	+	

*, †: Signify the same as in Table II.

Separation of Group Carbohydrate (Polypeptide-polysaccharide Complex) from the Mucus Fractions

The procedure was the one hitherto applied by us.

1) Scission of the carbohydrate with 1 *N* NaOH from Frs. I, II and IV at room temperature.

3 g. of Fr. I were taken up in 90 cc. of 1 *N* NaOH and shaken at 20°C for 24 hours. The insoluble part was rejected. The brown solution was acidified with glacial acetic acid to about pH 4.5, and the bulky deposit was centrifuged and washed with very dilute acetic acid of the pH above. The supernatant and washing were dialyzed together against running tap water till neutral, and after straining off of some precipitate, the filtrate was distilled in vacuo to about 20 cc., precipitated with 10 volumes of alcohol with subsequent washing with alcohol and dried. The crop at this stage was 0.9 g. The crude product was then extracted with a 5 cc. and a 4 cc. portion of water and to the clear solutions were added 18 volumes of glacial acetic acid, whereby a voluminous precipitate occurred. This mixture as such was shaken for 3 days. The centrifugate was washed with two 20 cc. portions of glacial acetic acid and three 20 cc. portions of alcohol and dried in a vacuum desiccator over H_2SO_4. 0.38 g. of a white powder was given. Next, it was dissolved in 25 cc. of water (A small part remaining undissolved was discarded), and 8 cc. of 10% NaOH and 3 cc. of 12.5% $CuSO_4 \cdot 5H_2O$ were added in sequence under agitation. The deposit was washed with four 20 cc. portions of 3% NaOH and dissolved in 3 cc. of 10% HCl (A small insoluble part was removed), then the solution was

precipitated with 10 volumes of alcohol, thoroughly washed with alcohol and dried. This $CuSO_4$-alkali process was repeated over again. The product here was dissolved in 100 cc. of water and freed from copper completely by electrodialysis. Followingly, the solution was condensed, precipitated by the aid of alcohol, and after washing with alcohol, dried similar to above. A white powder—**Ch.** $\mathbf{I_{20}}$—was given. Yield, 0.3 g. Biuret negative; Molisch and the indirect Osaki-Turumi strongly positive.

0.09 and 0.33 g. respectively of carbohydrate preparations corresponding to the one above were obtained from 1 g. of Fr. II and 2 g. of Fr. IV. They will be referred to as **Ch.** $\mathbf{II_{20}}$ and **Ch.** $\mathbf{IV_{20}}$ respectively.

2) Scission of the carbohydrate from Fr. I by means of 1 *N* NaOH at a lower temperature

3 g. of Fr. I were cleft with 60 cc. of 1 *N* NaOH by placing in an ice chest (0–3°C) for 2 days with occasional shaking. The succeeding operations were the same as in 1), excepting that the treatment with NaOH and $CuSO_4$ was effected at below 8°C. The final product (a white powder)—**Ch.** $\mathbf{I_0}$—amounted to 0.4 g. Its qualitative tests resulted as in the above products.

3) Scission of the carbohydrate from Fr. I by means of 1 *N* NaOH at a higher temperature

The alkaline cleavage was made at 30–35°C. The preparation procedure was otherwise similar as described in 1). 0.2 g. of a white powder was yielded from 3 g. of the mucus fraction started with — **Ch.** $\mathbf{I_{30}}$. Qualitatively it was identical with the products in 1) and 2).

4) Scission of the carbohydrate from Fr. I by the aid of trypsin

2 g. of Fr. I were taken up in 100 cc. of water and 0.6 g. of trypsin Kahlbaum was added. After further addition of 1% Na_2CO_3 to pH 9.0, the mixture was maintained at 37°C, readjusting the pH to the original from time to time. Toluene was used as a preservative. Incubation was continued for 60 days and the yellow-brown solution was freed from toluene by aeration, diluted to 200 cc. and acidified to pH 4.5 by addition of glacial acetic acid in drops. The deposit was centrifuged and washed with two 30 cc. portions of very dilute acetic acid of pH 4.5. The centrifuged supernatant and washings were dialyzed against running tap water till nearly neutral and distilled in vacuo to about 30 cc. The condensate was precipitated with 10 volumes of abs. alcohol, wahsed with alcohol and dried. The crude product here (a grey powder) weighed 0.75 g. It was agitated in 5 cc. of water and the insoluble part was again treated with 4 cc. of water. The solutions were united and 140 cc. of glacial acetic acid were added. The mixture containing a deposit was stood at 6–10°C for 2 days with occasional shaking. The centrifugate was

washed with 20 cc. of water once and with 20 cc. of alcohol thrice and dried as above. 0.48 g. of a white powder was given. This was again taken up in 5 cc. of water (The solution was of pH 4.0) and freed from the insoluble residue by prolonged centrifugation and the insoluble washed with 3 cc. of very dilute acetic acid of pH 4.0. The solution and washing were precipitated with 10 volumes of NaCl-containing alcohol, and after washing with alcohol, dried. The substance thus given still contained a tiny amount of a contamination insoluble in the dilute acetic acid of pH 4.0, which was removed by centrifugation after redissolving the substance. The solution was precipitated with alcohol and dried to a constant weight in vacuo over H_2SO_4. The product (a white powder)—**Ch. I_{tr}**—weighed 0.43 g. It behaved in test-tube tests same as the foregoing carbohydrate preparations and was transparently soluble in 10% trichloroacetic acid.

Chemical and Serological Properties of the Group Carbohydrate Preparations

Paper chromatography of sugar components. It was carried out as above. 20 mg. were taken from each of the carbohydrate preparations and hydrolyzed with 2 cc. of 1 *N* H_2SO_4. The hydrolysate, almost liberated from H_2SO_4 with baryta, was condensed to about 0.2 cc. and after shifting the pH to 5.8, sampled taking a 0.1 cc. portion. As is shown in Fig. 2, the products after alkaline scission of Frs. I, II and IV at 20°C as well as that after alkaline scission of Fr. I at 30–35°C contained glucosamine, galactose and L-fucose, but those after alkaline scission at 0–3°C and after tryptic digestion chondrosamine beside those sugars.

Analysis. The figures obtained are shown in Table IV. **Ch. TM** denotes a carbohydrate preparation separated from the total mucus after alkaline treatment at room temperature. It is noticeable that the L-fucose contents and iodine values of Chs. I_o and I_{tr} were less than the corresponding contents and values of the other carbohydrate preparations which were devoid of chondrosamine. The quantitative relations of the sugar components suggest that the latter preparations are producible by splitting off of approximately 4 molecules of chondrosamine or chondrosamine and glucosamine together and of galactose and one molecule of L-fucose from the former.

Group specificity. The results of assay processed similar to above are illustrated in Tables V–VI. Chs. TM, I_{20}, II_{20} and IV_{20} were inhibitory to A erythrocyte isoagglutination at a dilution of $1 : 8 \times 10^5$. Ch. I_{30} was of much lower potency, but Chs. I_o and I_{tr} the most potent compared with Fr. III. All the carbohydrate preparations lacked anti-β potency. As to E activity, Chs. TM, I_{20}, II_{20} and IV_{20} resembled Frs. I and II. Ch. I_{30} was less active than any of the mucus fractions, but Chs. I_0 and I_{tr}

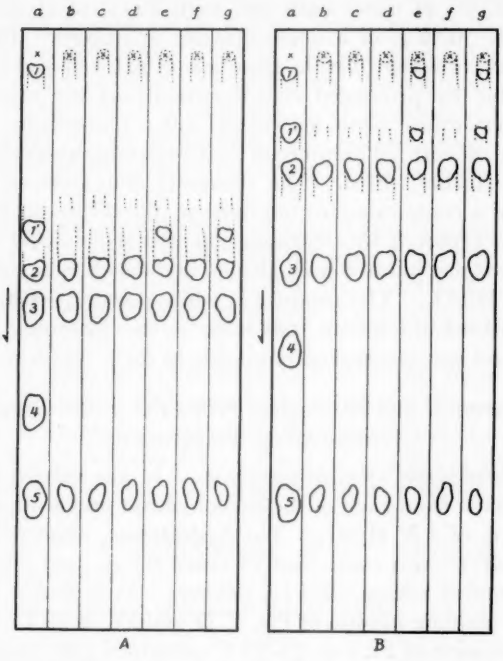

Fig. 2. Diagrammatical reproductions of the sugar chromatograms of the carbohydrate preparations, developed with (A) butanol-pyridine-water (5 : 3 : 2) and (B) *n*-butylacetate-acetic acid-ethanol-water (3 : 2 : 1 : 1). Temperature and duration of test 23–26°C and 24 hrs. respectively. Indicator aniline hydrogen phthalate.

a : reference run, b : Ch. I_{20}, c : Ch. II_{20}, d : Ch. IV_{20}, e : Ch. I_{o}, f : Ch. I_{30}, g : Ch. I_{tr}. 1 and 1′ : chondrosamine sulfate, 2 : glucosamine sulfate, 3 : galactose, 4 : mannose, 5 : L-fucose.

matched Fr. IV the most active fraction.

Summary

1. Pig stomach mucus was divided into four fractions including mucus-mucin of Sinokawa. The fractions contained hexosamine (glucosamine and chondrosamine altogether), galactose, L-fucose and mannose in molecular proportions almost coinciding mutually among them.

2. Three of the fractions including the mucus-mucin, which had been yielded comparatively largely, were subjected to alkaline scission at 20°C of the carbohydrate from the major part of the amino acid grouping. The preparations from the different fractions were similar in composition (They

TABLE IV

Composition of the Carbohydrate Preparations from Total Mucus and its Fractions of Pig Stomach

Figures in parentheses express molecular weights calc. from iodine values.

Analysis	In per cent							In equivalents per equivalent weight						
	Ch. TM	Ch. I_{20}	Ch. II_{20}	Ch. IV_{20}	Ch. I_o	Ch. I_{30}	Ch. I_{tr}	Ch. TM	Ch. I_{20}	Ch. II_{20}	Ch. IV_{20}	Ch. I_o	Ch. I_{30}	Ch. I_{tr}
N*	4.1	4.0	4.0	4.2	3.9	4.2	4.8	1.23	1.20	1.20	1.26	1.17	1.25	1.44
Hexosamine†	42.7	42.7	42.8	42.6	42.7	42.9	42.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Galactose‡	34.8	34.7	34.5	34.6	34.7	35.4	34.6	0.81	0.81	0.80	0.81	0.81	0.82	0.81
L-Fucose§	14.2	13.8	14.0	14.5	12.1	13.5	12.4	0.36	0.35	0.36	0.37	0.31	0.34	0.31
Acetyl¶	10.0	9.9	10.1	10.0	10.5	9.3	10.4	0.98	0.97	0.98	0.98	1.02	0.92	1.02
Ash//	1.1	1.3	1.0	2.5	0.8	1.0	3.3							
Iodine value**	5.7 (3700)	5.6 (3780)	5.7 (3700)	5.7 (3700)	3.8 (5570)	5.8 (3650)	3.9 (5430)							

*, †, ‡, §, // Signify the same as in Table I. ¶ Suzuki[7], ** Number of cc. of 0.1 *N* iodine used by 1 g. of substrate.

TABLE V

Occurrence of Agglutination of A Erythrocytes by Normal Serum, treated with the Carbohydrate Preparations

The same B serum as that for the assay in Table II was employed. − : no agglutination, ± : faint aggl., + : slight aggl., ++ : marked aggl.

Carbohydrate preparation	Dilution of substances (1 :)													
	2.5×10^4	5×10^4	10^5	2×10^5	4×10^5	8×10^5	1.6×10^6	3.2×10^6	6.4×10^6	1.28×10^7	2.56×10^7	5.12×10^7	1.024×10^8	∞
Ch. TM	−	−	−	−	−	−	±	+	+	++	++			++
Ch. I_{20}	−	−	−	−	−	−	+	+	+	++	++			
Ch. II_{20}	−	−	−	−	−	−	+	+	+	++	++			
Ch. IV_{20}	−	−	−	−	−	−	±	+	+	++	++			
Ch. I_o	−	−	−	−	−	−	−	−	−	−	−	±	+	
Ch. I_{30}	−	−	+	+	+	+	+	++	++	++	++			
Ch. I_{tr}	−	−	−	−	−	−	−	−	−	−	−	±	+	

contained glucosamine, galactose and L-fucose as sugar components) and group activity as the corresponding preparation from the total mucus.

3. With other portions of the mucus-mucin were prepared the carbohydrate after alkaline treatment at different temperatures and after tryptic digestion. The substances obtained after alkaline scission at 0–3°C and after tryptic digestion possessed higher molecular weight and Group A

TABLE VI

Occurrence of Agglutination of O Erythrocytes by an Eel Serum treated with the Carbohydrate Preparations

The eel serum employed was the same as that for the assay in Table III. − : no agglutination, ± : faint aggl., + : slight aggl.

Carbohydrate preparation	Dilution of substances (1 :)						
	4×10^4	8×10^4	1.6×10^5	3.2×10^5	6.4×10^5	1.28×10^6	∞
Ch. TM	−	−	−	±	+	+	+
Ch. I_{20}	−	−	−	±	+	+	
Ch. II_{20}	−	−	−	+	+	+	
Ch. IV_{20}	−	−	−	±	+	+	
Ch. I_o	−	−	−	−	−	+	
Ch. I_{30}	−	−	+	+	+	+	
Ch. I_{tr}	−	−	−	−	−	+	

activity than those in 2. and moreover contained chondrosamine as an additional sugar component. The substance after alkaline cleavage at 30–35°C was found to resemble those in 2. regarding composition and molecular weight, but was less group potent particularly with respect to inhibition of isoagglutination of A erythrocytes.

4. Some deductions were expressed.

Through the Grant Committee for Scientific Researches was given a grant from the Education Department in aid to us. H. Masamune

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Biochemical Studies on Carbohydrates

CLXIX. A $HgCl_2$-test and a Means to differentiate Free Sugars, N- and O-Glycosides and Schiff Bases from one Class to Another

By

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(Received for publication, September 13, 1953)

N-Glycosides in solution are labile at the acid reaction and undergo hydrolysis. But they are pretty stable at the alkaline reaction and can not reduce $HgCl_2$ at pH 7.5–8.0 contrary to free sugars, when examined under conditions below. The former show the positive Fehling and Benedict reactions like the latter and unlike O-glycosides. Schiff bases resemble N-glycosides in the above respects excepting that they don't give Benedict reaction. Thus, the properties above of those classes of compounds are available for their mutual differentiation.

Technique of the $HgCl_2$-test

Reagents. $HgCl_2$ solutions. The sat. $HgCl_2$ is diluted 6-fold with water and divided into two portions and 1 *N* NaOH is added to one of the portions to pH 7.5 and to the other to pH 8.0. After standing for 30 minutes, the precipitates are centrifuged off and the clear supernatant are used for the assay. The solutions must be freshly prepared before use.

Procedure. Two portions of a solution of an unknown are adjusted to pH's 7.5 and 8.0 separately with 1 *N* NaOH and 0.1 cc. of each is pipetted into a 10.6 cm. × 1.1 cm. test tube, followed by adding 0.6 cc. of the $HgCl_2$ reagents at pH 7.5 and at pH 8.0 respectively. The mixtures are stoppered with cotton, heated in boiling water for exactly 40 minutes and cooled in running water. An occurring precipitate is centrifuged, washed with 2 cc. of water and examined with the microscope. Presence of crystals in small needles, which are characteristic of Hg_2Cl_2 and blackened by NH_3 (1 drop of 10% NH_3 is used), shows the positive reaction.

Results in Free Sugars, N-Glycosides and Schiff Bases

Glucose, galactose, lactose, glucuronic acid and N-acetylglucosamine as little as below gave the positive results.

Glucose and galactose	0.03 mg. at pH's 7.5 and 8.0
Lactose	0.08 mg. „ „ „ „ „
Glucuronic acid	0.035 mg. „ „ „ „ „
N-Acetylglucosamine	0.06 mg. at pH 7.5; 0.04 mg. at pH 8.0

In the cases of first three sugars, the reaction mixture turned more alkaline on heating, but in those of the other sugars the pH of the mixture decreased even to somewhat below the original after transitory increase.

3 mg. of N-glycosides and Schiff bases did not reduce mercuric chloride, but did when previously stood in water or acidulated water for 4 hours. In Tables I and II are illustrated the examples. Table I shows also the behaviour of the compounds toward Fehling and Benedict reagents. Schiff bases employed were synthesized as follows:

TABLE I

Behaviour of N-Glycosides and Schiff Bases toward the $HgCl_2$-, Fehling and Benedict Reagents

−: negative reaction, +: slightly positive r., ++: markedly positive r.

	Substrate	Test			
		with $HgCl_2$ solution* at		Fehling†	Benedict‡
		pH 7.5	pH 8.0		
N-Glycoside	Aniline-α-glucoside	−	−	++	+
	„ -β-galactoside	−	−	++	+
	„ -β-lactoside	−	−	+	+
	p-Aminobenzoic acid-β-glucoside	−	−	++	+
	„ -β-xyloside	−	−	++	+
	Glycineethylester-β-glucoside	−§	−§	++	+
	„ -β-galactoside	−§	−§	++	+
	„ -β-lactoside	−§	−§	++	+
Schiff base	Acetaldehyde-phenylhydrazone	−	−	+	−
	„ -*p*-aminobenzoic acid	−	−	+	−
	Furfural-glycine (barium salt)	−	−	+	−
	„ -tyrosine (barium salt)	−	−	+	−

* 3 mg. of the substrates were subjected to each test. † 0.1 cc. of solutions containing 0.5 mg. of an N-glycoside or a Schiff base were heated together with 2 cc. of the reagent in boiling water. ‡ 0.1 cc. of solutions containing 0.5 mg. of an N-glycoside or 2 mg. of a Schiff base were heated together with 2 cc. of the reagent in boiling water. § pH decreased on heating by about 0.3.

Acetaldehydephenylhydrazone, after Bamberger and Pemsel[1].

Acetaldehyde-*p*-aminobenzoic acid. A solution of 0.5 g. of *p*-aminobenzoic acid in 30 cc. of ether was chilled in an ice-salt mixture and after

TABLE II

A Solution of N-Glycosides in Water and Those Acidulated were stood at Room Temperature prior to the $HgCl_2$-test

The solutions after standing were adjusted to pH 7.5 or to pH 8.0 and the corresponding $HgCl_2$ reagents were added. − : negative reaction, + : slightly positive r.

Substrate (N-glycoside) \ Initial pH of test mixture	Solutions in water*				Solutions acidulated† and stood for 4 hrs.	
	immediately after dissolution		after standing for 4 hrs.			
	7.5	8.0	7.5	8.0	7.5	8.0
Aniline-α-glucoside	−	−	+	+		
" -β-galactoside	−	−	+	+	+	+
" -β-lactoside	−	−	−	−	+	+
p-Aminobenzoic acid-β-glucoside	−	−	+	+		
Glycineethylester-β-glucoside	−	−	+	+		
" -β-galactoside	−	−	+	+		
" -β-lactoside	−	−	+	+		

* pH of the solutions about 6.6. It increased by about 0.2 on standing except in the case of aniline-β-lactoside. † Acidulated with 1 *N* HCl to pH 5.6. The pH of the solutions increased on standing.

addition of 0.3 cc. of acetaldehyde under continuous cooling, shaken, followed by standing in an ice chest. Crystals in needles that had occurred were filtered off and washed with ether. F.P. 113°C. It contained no crystal water. N (micro Kjeldahl): Found 8.37%; calc. for $C_9O_2H_9N$ 8.59%.

Barium salt of furfuralglycine, after Bergmann *et al.*[2]

Barium salt of furfuraltyrosine. 0.2 g. of tyrosine and 0.2 g. of crystalline baryta were dissolved together in 12 cc. of water by warming and allowed to cool and 0.05 cc. of furfural was added. On standing the mixture in an ice chest, thin needles precipitated, which were separated on a filter and after washing with cooled water and alcohol, dried in air. F.P. 265°C. No crystal water was present. N (micro Kjeldahl): Found 4.31%; calc. for $C_{28}H_{24}O_8N_2Ba$ 4.29%.

The preparation procedures of the N-glycosides in the tables exclusive of those seen in literature will be described in a forthcoming article regarding optical behaviour of N-glycosides in solution.

Through the Grant Committee for Scientific Researches was given a grant from the Education Department to defray the expenses of this work.

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The Tohoku Journal of Experimental Medicine, Vol. 60 No. 2, 1954

Studies on Tuberculous Bacilluria in Bone and Joint Tuberculosis

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(Received for publication, September 14, 1953)

Tuberculous bacilluria in patients with extrarenal tuberculosis has been investigated by many foreign workers, who report higher incidences of tuberculous bacilluria especially among patients suffering from bone and joint tuberculosis. Literature in this field is scanty in Japan. Recently I have made examinations for tuberculous bacilluria on 203 patients with bone and joint tuberculosis at the Tamaura National Sanatorium. The scope of the present paper is to report and discuss the results obtained.

Experimental

Technique of the culture test of urine for tubercule bacilli

Spontaneous urine collected early in the morning was terated by Oka-Katakura's zinc sulfate method[1]; given amounts of the sediment were transferred to three tubes containing Oka-Katakura's culture medium[2] and incubated for culture of tubercle bacilli. The positive cases obtained were graded according to the number of colonies found as follows: +, 1–20 colonies; ++, 21–50; +++, 51–200; and +++, over 200. If the urine was examined at regular intervals for at least two months and found to be free from tubercle bacilli, the case was considered negative.

Results

Culture tests for tuberculous bacilluria on patients with bone and joint tuberculosis

Culture tests for tuberculous bacilluria on patients with bone and joint tuberculosis and without remarkable bladder symptoms gave the following results: (1) In the first year of examination, where three weekly tests were made upon each patient, 23 patients (17.6%) out of 131 were positive; and (2) in the second year and later, where consecutive monthly tests were made (over a period as long as 14 months in some cases), 37 patients (24.0%)

out of 154 were positive. Combining the results from both groups (some patients were duplicated in both groups), there were obtained 50 positive cases (24.6%) out of a total of 203. From Table I, in which are listed the results reported by various authors it will be seen (1) that the rate of occurrence of tuberculous bacilluria as obtained in the present investigation is relatively high; and (2) that the rate in bone and joint tuberculosis is higher than in pulmonary tuberculosis, as pointed out by Harris[5], McClelland[9] and others. It is to be noted that Yokoyama's data, listed in the table, on patients with pulmonary tuberculosis are the ones obtained by examinations in parallel with my examinations. In view of Band's high value (21.3%) in pulmonary tuberculosis, the rate of positiveness might depend also upon the cases to be tested, the method and the interval of testing.

TABLE I

Occurrence of Tuberculous Bacilluria Collected from Literature

Author		Total cases	Bacilluria	Percent	Extrarenal lesion
Brown	(1915)*	104	11	10.0	Pulmonary
Hobbs	(1923)*	100	6	6.0	Pulmonary
Spitzer et al.	(1927)†	103	0	0	Pulmonary
Harris	(1929)	110	25	22.7	Bone and joints
Dimtza	(1932)*	183	8	4.4	Extrarenal
Band and Munro	(1935)*	174	25	14.4	Pulmonary
Saenz et al.	(1935)†	100	0	0	Pulmonary
Kjaer	(1937)†	300	33	11.0	Extrarenal
Sato	(1937)	487	34	6.6	Pulmonary
Rosencrantz	(1940)	200	14	7.0	Pulmonary
Band	(1942)*	300	64	21.3	Pulmonary
Ordway & Medlar	(1942)	276	17	6.1	Pulmonary
Yokoyama	(1952)	448	30	6.7	Pulmonary
Present series		203	50	24.6	Bone and joints

* Cited by Band (1948)[3]. † Cited by Ordway & Medlar (1942)[4].

a) *Relation of the incidence of tuberculous bacilluria with those of albuminuria and pyuria.* Pyuria was found at least microscopically in all 50 cases with tuberculous bacilluria, whereas about half of the cases did not accompany albuminuria. Tubercle bacilli were not proved in about half of the patients with albuminuria or pyuria. Fain[10], Lieberthal[11], Wildbolz[12] and others, found cases with tuberculous bacilluria but without pyuria, which is contrary to my findings. Colby[13] states: "In the presence of albuminuria, guinea pigs have been positive before the appearance of blood and pus in the urinary sediment frequently enough so that we have come to regard the constant presence of even small amounts of albumin as a very early indication of renal involvement in patients with bone tuberculosis." In my study, about half of the cases with albuminuria were positive for tubercle

bacilli, and all these positive cases were found microscopically to be accompanied by pyuria. Even if examination of many microscopic fields of a preparation revealed only one to several pus cells, the case was taken to be with pyuria. Noseworthy was it that, on inspecting individual cases, there were no cases with tuberculous bacilluria accompanied by albuminuria but not by pyuria.

b) *Relation of the number of colonies of tubercle bacilli to that of pus cells* (Table II). The number of pus cells was counted on fresh smears prepared in a fixed manner from the sediment obtained by centrifuging the urine generally at 3000 rpm for 10 minutes. When the number of pus cells found in a microscopic field was only a few, the case was classed as "scanty"; when from several to atoub 20, as "moderate"; and when more than 20, as "abundant". It will be seen from Table II that in most cases with scanty pus cells the colonies of tubercle bacilli were only a few in number (+). This might be suggestive of a parallelism between the number of colonies and that of pus cells, as maintained by Harris,[5] but the table shows that this is not necessarily the case, for there are exceptions, for instance, 9 of the 34 cases with scanty pus cells had many bacillus colonies (4 ## and 5 ### cases).

TABLE II

Relation of the Number of Colonies of Tubercle Bacilli as Developed on Culture with the Presence of Pus Cells in the Urine, in 50 Case with Tuberculous Bacilluria

		Number of pus cells			Total
		Scanty	Moderate	Abundant	
Number of tubercle bacilli	+	21	2	1	27
	##	1	1		2
	###	4	2		6
	####	5	8	2	15
Total		34	13	3	50

c) *Incidence of tuberculous bacilluria and the site of lesions* (Table III). The incidence in patients with spinal caries concurrent with other bone and joint tuberculosis was as high as 31.7 per cent, while the incidence in spinal caries and that in joint tuberculosis were nearly equal and relatively low.

The incidence of the bacilluria in 128 patients with vertebral caries is found to have been higher in the order of dorsal (34.4%), dorsal plus

TABLE III

Occurrence of Tuberculous Bacilluria and the Site of Lesions in Cases with Bone and Joint Tuberculosis

Site of lesion	Number of cases	Positive case	
		Number	Rate (%)
Spine	128	29	22.6
Spine and other bone or joint	41	13	31.7
Joint	33	7	21.2
Sternum and rib	1	1	
Total	203	50	24.6

lumbar (25.5%), and lumbar vertebra (16.2%). Hemprich,[14] Moccia,[15] and others have pointed out that many patients with caries of the lumbar vertebra accompany urogenital tuberculosis, but tuberculous bacilluria may hardly be said to have occurred especially frequently in lumbar caries.

d) *Incidence of tuberculous bacilluria in groups with single and multiple lesions.* The incidence in 61 cases of the group with multiple lesions was as high as 31.1 per cent, although it is found in 21.8 per cent out of 142 cases of the group with single lesion.

e) *Incidence of tuberculous bacilluria in groups with and without tuberculous complications other than bone and joint tuberculosis.* The incidence was as high as 36.1 per cent in 119 cases of the group of the tuberculous complications, whereas 8.0 per cent out of 84 cases of the group without other complications. When 97 patients complicated with pulmonary tuberculosis, which is the most common of tuberculous complications other than bone and joint tuberculosis, are classified according to the degree of pulmonary lesions appearing on thoracic roentogenograms,[16] the incidence of the bacilluria is as shown in Table IV, where it will be seen that the incidence was higher in more extended lesions.

If the cases with tuberculous bacilluria are grouped according to types of pulmonary lesions after Rehberg's pathologicoanatomic classification[17] (Table V), the incidence will be found to have been relatively high in primary tuberculosis, disseminative tuberculosis and tuberculous pulmonary infiltration.

Surgical renal tuberculosis

Upon the above-mentioned 50 cases with tuberculous bacilluria were carried out various urological examinations; the results are:

Intravenous pyelography. Upon 18 of the 50 patients pyelogarphy

TABLE IV

Occurrence of Tuberculous Bacilluria and the Degree of Lesions of Tuberculous Pulmonary Complications

Lesion	Number of cases	Positive case	
		Number	Rate (%)
Minimal	61	20	32.7
Moderately advanced	28	10	35.7
Far advanced	8	5	62.0
Total	27	35	36.0

TABLE V

Occurrence of Tuberculous Bacilluria in Eight Groups of Tuberculous Pulmonary Complications

	Number of cases	Positive case	
		Number	Rate (%)
Primary tuberculosis	25	10	40.0
Disseminative tuberculosis	6	3	50.0
Tuberculous pulmonary infiltration	7	3	42.8
Cheesy pneumonia and bronchipneumonic tuberculosis	1	1	
Nodular-cirrhotic tuberculosis	28	8	28.5
Bronchogenic progressive mixed phthisis	10	2	20.0
Cirrhotic pulmonary tuberculosis	15	5	33.3
Pleurisy	5	3	
Total	97	35	36.0

could not be performed because of serious general physical conditions or for other reasons. Pyelography performed upon the remaining 32 patients brought out pictures normal or nearly normal bilaterally in 16 of them, which are regarded as subclinical stage, and pictures abnormal in varying degrees unilaterally or bilaterally in the remaining 16 patients which are said as surgical renal tuberculosis.

The relation of the pyelographic findings to the number of colonies of tubercle bacilli and of pus cells is shown in Table VI. It will be seen that (1) the colonies were numberous in many of the 16 patients with surgical renal tuberculosis; (2) of patients with subclinical renal tuberculosis, three produced ##-colonies, and four ###-colonies; and (3) pus cells were scanty in most of the subclinical cases, and moderate or abundant

TABLE VI

Relation of Pyelographic Findings with the Number of Tubercle Bacillus Colonies and of Pus Cells in 32 Cases with Tuberculous Bacilluria

Pyelographic finding	Number of colonies of tubercle bacilli				Presence of pus cells		
	+	++	+++	++++	Scanty	Moderate	Abundant
Both sides: normal or nearly normal	8	1	3	4	14	3	
One side: normal or nearly normal Other side: pathologic	4	1	1	5	5	4	1
One side: slightly abnormal Other side: remarkably pathologic			1	3		3	1
Both side: pathologic				2		1	1

in many of the surgical cases.

Cystoscopy, chromocystoscopy, and catheterization on patients with surgical renal tuberculosis. Cystoscopy made upon 10 of the 16 patients revealed that the mucosa of the bladder was normal in one, and slightly red in the other nine, there being no cases with evidence conclusive of tuberculous lesions such as tubercles and ulcers.

Chromocystoscopy also was performed upon these 10 patients, with the results as follows: The dye efflux was bilaterally normal in five, unilaterally slightly weakened in one, unilaterally lacking in three, and bilaterally slightly weakened in one.

Further, in six of the 10 patients the urine was collected for 25 to 30 minutes by bilateral ureteral catheterization, and examined for pus cells and tubercle bacilli, with the following results: As for pus cells, the urine was unilaterally positive in four, and bilaterally positive in two: as for tubercle bacilli, bilaterally negative in one, unilaterally positive in four, and bilaterally positive in one. The results of unilateral ureteral catheterization performed on three patients were as follows: The urine from the diseased kidney in one case and that from the "good" kidney in two cases were positive for both pus cells and tubercle bacilli. For the remaining one patient, ureteral catheterization failed.

Of the 10 patients upon which various examinations were made, only four were diagnosed to have unilateral renal tuberculosis and judged as having ideal indications for operation, while the other six were conclusively or inconclusively diagnosed to have bilateral renal tuberculosis, and four of them judged as relative indication for operation.

Nephrectomy: Six patients (2 with ideal and 4 with relative indications for operation) were subjected to nephrectomy, which revealed

tuberculous pyelonephritis in one, tuberculous cavitation in four, and tuberculous pyonephrosis in one. Upon the two patients judged as ideal indication, partial resection of the upper pole of the kidney was performed: In one of them tuberculous ulceration was perceived in the papilla-calix area, while in the other no tuberculous lesion was observed either macroscopically or histopathologically.

In the four patients operated on under ideal indications, the urine became negative for tubercle bacilli after nephrectomy or partial resection. On the contrary, in four cases, having relative indications for operation, tubercle bacilluria persisted till after the operation against the succeeded chemotherapy.

Follow-up observations in the course of disease in eight patients with surgical renal tuberculosis but not operated. The course of disease was followed up with two unoperated patients who had been judged to be with bilateral lesions and six who had undergone no urological examination for various reasons, with the following results: In four patients the number of colonies of tubercle bacilli in the urine increased from ## to ### of from + to ### in 14 months; in one, the number remained unchanged during three months of observation; one patient removed to another hospital; and the remaining two died under a complication of tuberculous meningitis.

Subclinical renal tuberculosis

Upon 11 of 16 patients judged as subclinical renal tuberculosis, cystoscopy was performed, with the results that the mucosa of the bladder was normal in seven, slightly red in four, changes determinable to be tuberculous lesions such as tubercles or ulcers were not found in any of the cases.

Chromocystoscopy made upon these 11 patients resulted in that unilateral slight weakness of the dye efflux was found only in one case, all the others being bilaterally normal in the efflux.

Bilateral ureteral catheterization was carried out on 10 of these patients. It was found that the kidney urine was unilaterally microscopic positive for pus cells in two, bilaterally positive in one, but bilaterally negative in the remaining seven patients. As for tubercle bacilli the urine was bilaterally positive in one, and bilaterally negative in the remaining nine patients.

Unilateral ureteral catheterization was carried out on one of the 11 patients, but the kidney urine was negative for both pus cells and tubercle bacilli.

One patient of these 11 died of tuberculous meningitis about four months after the examinations. No tuberculous lesions are found histopathologically on both kidneys, though serial sections were not made.

The course of the disease was followed in 15 patients with subclinical renal tuberculosis for a period from 14 to 26 months; one died.

At pyelography, cystoscopy, chromocystoscopy and examination by means of ureteral catherization, no remarkable chane was found in the patients.

During the above-mentioned periods of examinations 10 to 26 intermittent tests for both tubercle bacilli and pus cells in the bladder urine were performed upon each of the 15 patients, and the cases were classed into three groups according to the frequency of occurrence of tuberculous bacilluria, as shown in Table VII: (1) the first group, including eight patients with frequent occurrence of positive urines; (2) the second group, including eight patients whose urines became positive about once a year; and (3) the third group, including two patients whose urines had been negative for more than one year after the first test. As for the occurrence of pus cells in the ruine, it was found that they made their appearance frequently in the first, occasionally in the second, and scatteringly and sparingly in the third group.

It was pointed out first by Medlar,[19] then by Harrs,[5] Band[18] and others[20] that the earliest minor lesions in the renal cortex are curable in many cases, and then tuberculous bacilluria may disappear. The two cases of the third group may be regarded as clinical cure.

18 patients without urological examinations

In 18 of the 50 patients with tuberculous bacilluria on whom intravenous pyelography or other urological examinations could not be made because of their unfavorable general physical conditions or their circumstances, positive urine occurred only once or twice upon frequent examinations. Two of the patients died of tuberculous meningitis; of these two, one patient whose urine was strongly positive (##) at the first test was found at autopsy to have had a cavitation form of renal tuberculosis on both sides; upon the other patient autopsy could not be carried out.

Summary

1. Upon a total of 203 cases with bone and joint tuberculosis, culture test for tubercle bacilli in the urine was carried out at regular intervals over a long period of time, with the results that the bacilluria took place in 50 cases (24.6%). Cases with the bacilluria were found, at least microscopically, to accompany pyuria.

2. 32 cases with bacilluria were divided from the findings in the pyelograms into "surgical renal tuberculosis" of 16 cases and "subclinical renal tuberculosis" of 16 cases.

3. Among 16 patients with surgical renal tuberculosis, four were

TABLE VII

Variations in Occurrence of Tubercle Bacilli in the Urine of 15 Patients with Subclinical Renal Tuberculosis

	Case No.	Age	Sex	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26 Month
1st group	21	46	♂	##	−	##	−	−	−	+	−	+	##	##	##	+	+												
	22	25	♂	##	##	##	##	−	+	−	−	+	−	−	−	+	+												
	24	29	♀	##	##					+						−	##	+	−	+	−	−	−	−	−				
	26	29	♀	−	−	+	−	−	−	−	−	−	−	##	−	−	−												
	27	44	♀	+	−	−	−	−	−	−	+	+	−	−	−	−	−												
	28	31	♂	−	+	−	−	−	−	−	−	−	−	+	−	−	−												
	29	32	♂	−	−	−	−	−	+	−	−	−	−	+	−	−	−												
	31	30	♂	##												−	−	−	−	−	+	−	−	##	−	+	−	−	−
2nd group	18	34	♂	+	−	−	−	−	−	−	−	−	−	−	−	−	##	−	−	−	−	−	−	−					
	19	24	♂	##	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−						
	23	34	♂	##	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	
	30	36	♀	−	−	−	−	−	−	−	−	−	−	−	−	+	−												
	32	36	♂	−	−	−	+	−	−	−	−	−	−	−															
3rd group	20	35	♂	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	25	34	♀	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			

judged as unilateral and six as bilateral by means of urological examinations, though these examinations were not performed in the remaining six cases.

4. Upon 15 of the 16 patients with subclinical renal tuberculosis (the remaining one died), observation of the occurrence of tuberculous bacilluria was continued over a period from 14 to 26 months, with the results that the urine remained constantly free from tubercle bacilli in eight, mostly negative and infrequently positive in five, and constantly negative in the remaining two patients.

5. In 18 of the 50 patients with tuberculous bacilluria an exact examination could not be made because of various reasons.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Studies on the Development of Surgical Renal Tuberculosis

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(Received for publication, September 14, 1953)

It has been well established by Wegelin-Wildbolz,[1] Koike,[2] and others that surgical renal tuberculosis begins at the papilla-calyx area. Though the process of appearance of this primary lesion at the papilla-calyx area was not clear at first, it has recently been generally accepted among American and British workers, dating back to the work of Medlar,[3] that (1) tubercles are formed first in the cortex by tubercle bacilli carried by the blood stream to the kidney; (2) these early tubercles have a strong tendency to heal to scars, thus usually becoming harmless; (3) but if a tubercle continues to grow under certain circumstances and breaks into a renal tubucle, the discharged bacilli descend along the tubule to the papilla-calyx area, where they give rise to the formation of a lesion, surgical tuberculosis being thus developed (Lieberthal,[4] Band,[5] Lowsley and Kirwin,[6] Dukes[7]). This hypothesis is based upon the results of studies by Harris[8] and others on tuberculous bacilluria of patients with renal tuberculosis at sanatoriums and upon those obtained by Medlar and his school[9] from experiments on laboratory animals and from histological studies of kidneys of patients dying of renal tuberculosis. It is difficult to verify this hypothesis by histologic examination of operative removed kidneys of patients with surgical renal tuberculosis. If, however, out of many clinically excised tuberculous kidneys are selected those specimens in which the lesions are only localized and the remaining relatively "normal" areas are large, and then the latter "normal" areas are examined by the serial section method, it might be possible to obtain findings that afford some basis for this hypothesis. In view of this posibility, careful microscopical examination of serial sections was made upon relatively normal areas of three such specimens of kidney, with the results to be reported in the present paper.

Material and Method

Case 1. A 25 year old male with left renal tuberculosis. One cut

surfaces there was observed a caseous papillary lesion, 0.7×0.5 cm., on a middle minor calyx. On the contiguous mucosa of the pelvis, tubercles and a tuberculous ulcer were found in a small area. Macroscopically, other pelvis as well as major and minor calyces were nearly normal, there being detected subepithelial tubercle formation on a few lower calyces only microscopically. In the remaining renal parenchyma, only a few tubercles were found macroscopically. The whole kidney was divided into 73 blocks and a total of 31,322 sections were prepared.

Case 2. A 16 year old male with left renal tuberculosis. On the upper pole of the kidney there was observed a caseous papillary lesion, 0.6×0.6 cm.; no remarkable changes were found in the remaining renal parenchyma. The mucosae of upper major calyces were caseous over the whole surface; the upper half of the pelvis mucosa was affected by tuberculous ulceration, while no macroscopic changes were perceived on the mucosa of lower major calyces. The whole kidney was divided into 71 blocks, and a total of over 5,000 sections were prepared for closer examinations of relatively normal parts of the lower half of the kidney.

Case 3. A 32 year old female, with left renal tuberculosis. The tuberculous lesions were found to be more advanced than in the above two specimens. There were observed a cavitation, 1.5×1.2 cm., on the lower pole of the kidney, and remarkable tuberculous lesions over the whole surface of the lower half of the pelvis mucous membrane. Minor calyces situated above an upper major calyx showed a thickening due to a stricture at the cervical part of the latter, but no marked tuberculous lesions were observed. A total of over 4,000 sections were prepared for closer examinations of the relatively normal upper part of the kidney. A small number of sections were prepared also from several areas of the middle and lower portions of the kidney.

Staining of the secitons. Most of the sections thus prepared from the three kidney specimens were stained with hematoxylin and eosin. The remaining small number of sections were treated with Van Gieson's stain, Mallory's stain, Weigert's stain for elastic fibers, a stain for tubercle bacilli, or Bielschowsky's silver method.

Results

Results of examinations in Case 1.

1) The middle papilla-calyx area bearing the major lesion. The caseous lesion affected almost all the papillae and medulla and extended as far as the corticomedullary zone and medulla, being surrounded by tuberculous granulation tissue containing a large number of tubercles. From this lesion, restiform lesions being produced by round cell infiltration and sometimes accompanying a tubercle formation extended radially to

the surface of the kidney (Fig. 1). These changes were restricted to the

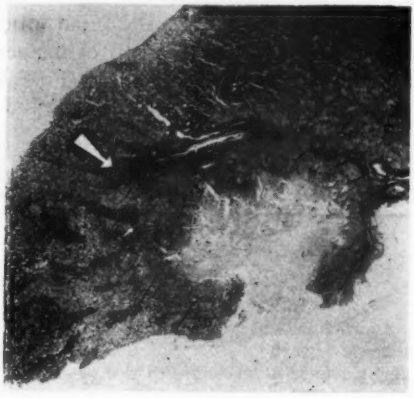

Fig. 1. Restiform lesions extending radially from a caseous lesion to the surface of the kidney. A tubercle is shown by the arrow.

renal lobe belonging to the affected papilla, there existing in the medulla and cortex 86 conglomerate tubercles of about 1 cm. in diameter (Fig. 2) and still more abundant " smallest giant cell tubercles " to be described later. Almost all these tubercles seem to have ensued pyelonephritically

Fig. 2. Conglomerate tubercles in restiform lesions.

from the caseous lesion, but whether there were mixed among them hematogenous tubercles that had become the origin of the caseous papillary lesion according to Medlar's hypothesis was not clear, due to the presence

of too large a number of tubercles there.

b) Lesions in the renal parenchyma of the relatively normal area. Macroscopically, the portions other than the renal lobe having the main lesion appeared nearly normal. Examination of the serial sections, however, revealed 58 tuberculous nodules in the latter (Table I and Fig. 3).

TABLE I

Tuberculous Nodules in the Relatively "Normal" Area of the Kidney

	Site / Lesion	Cortex			Renal column	Renal sinus	Total
		Renal surface	Intermediate resion	Corticomedullary zone			
Case 1	Epithelioid tubercle	0	2	0	0	0	2
	Giant cell tubercle	2	23	22	3	2	52
	Extinct conglomerate lesion	1	2	1	0	0	4
Case 2	Epithelioid tubercle	0	6	1	0	0	7
	Giant cell tubercle	3	0	0	0	0	3
Case 3	Epithelioid tubercle	2	1	0	0	0	3
Total		8	34	24	3	2	71

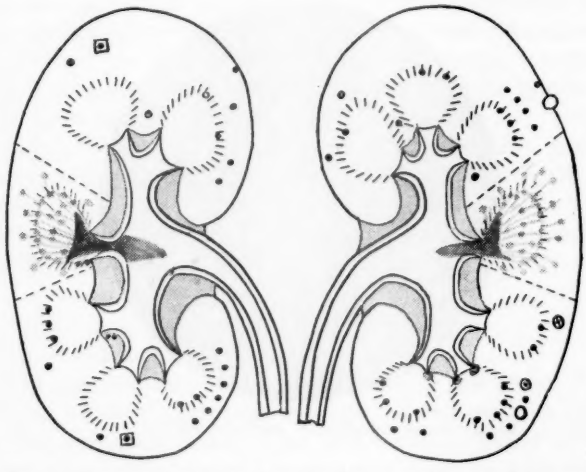

Fig. 3. The distribution of tuberculous lesions in Case 1. ⊕, 1st conglomerate lesion. ⊙, 2nd conglomerate lesion. ○, 3rd and 4th conglomerate lesions. ●, Smallest tubercles. ⊡, Epithelioid cell tubercles.

Fig. 4. The smallest giant cell tubercle. The arrow indicates a tubule.

A large majority (52) of these nodules consisted of one to four giant cells (Fig. 4), a few epithelioid cells and fibroblasts, and were surrounded by a layer of lymphocytes. They were of the order of magnitude of a glomerulus or smaller and will be referred to as " smallest giant cell tubercles " in this paper. There were found only two " epithelioid cell tubercules ", i.e. those smallest tubercles which were similar to the above but not found to contain giant cells upon examination of the serial sections. One of them was situated in the middle portion of the upper renal cortex, where both the calyx mucosa and papillae were intact; and the other was situated in the middle portion of the cortex, where a small caseous lseion was seen at a lower calyx niche; both these tubercles were situated apart from the glomerulus.

The distribution of these 52 smallest giant cell tubercles is shown in Table I, where it will be seen that only two tubercles were present in the renal sinus, while the remaining 50 were all found in the cortex or the renal columns. As for the relation of these 52 tubercles with the glomerulus, each of 18 tubercles laid close to a glomerulus or with the surrounding lymphocytes infiltrating into the glomerulus, and the remaining 32 were found isolated from the glomerulus. The smallest of these tubercles were all formed interstitially; the smallest ones lightly pushed the renal tubule and occasionally the glomerulus, while in a little larger ones the surrounding round cell infiltration extended as far as the renal tubule and glomerulus, but there was found no case in which a tubercle broke into these tubules or involved the glomerulus.

The two smallest giant cell tubercles as seen in the renal sinus were the ones that had been formed side by side in the same area contiguous

to the mucosa of the minor renal calyx on the lower pole of the kidney. On this portion of the mucosa there was observed a very slight extent of round cell infiltration, no definite tuberculous changes being demonstrated.

These changes, however, are regarded to have ensued from the calyx mucosa, for tuberculous nodules were found at the calyx niche of the adjacent papilla (Fig. 5).

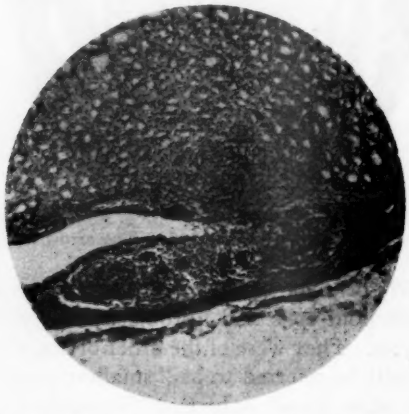

Fig. 5. A tuberculous lesion at a lower calyx niche found in Case 1.

All the smallest giant cell tubercles as found in the renal columns were situated on the upper pole of the kidney. No tuberculous lesion was detected on the mucosae of the major and minor calyces of this portion. Of the 47 cortex smallest giant cell tubercles, two were formed in close proximity to the renal surface, 22 in the cortex at the corticomedullary zone, and 23 in the central portion of the cortex, solitarily at places other than the renal lobe belonging to the main lesion in the middle part of the kidney. No tuberculous lesions were revealed on the minor renal calyces, mucosae, and papillae in all the portions of the kidney, with an exception of a part of the lower pole, nor were pyelonephritic lesions demonstrated in the area between the tubercles and the papilla-calyx area.

Besides the smallest giant cell tubercles mentioned above, there were found four relatively large tubercles, more than ten times as large as the glomerulus. The findings common to these tubercles were that they were much larger than the smallest giant cell tubercles, and that there was found a remarkable proliferation of the connective tissue fibers.

The first conglomerate lesion of tubercles extended from the corticomedullary zone of the lobe adjacent to the lobe having the main lesion to the middle portion of the cortex, and the papilla in this portion was

intact. The central part of this conglomerate lesion of tubercles consisted mainly of epithelioid cells and connective tissue fibers. This was surrounded first by the smallest tubercles having giant cells, then by a layer of lymphocytes.

The second conglomerate lesion (Fig. 6) was found in the cortex of the lower part of the kidney, in which a central caseous lesion of a small area was surrounded first by connective tissue fibers, then by tuberculous granulation or by tubercles, and finfilly by a layer of lymphocytes. In this neighborhood there were seen also scarred glomeruli. The papillary area in this part corresponded to the calyx-niche lesion shown in Figure 5.

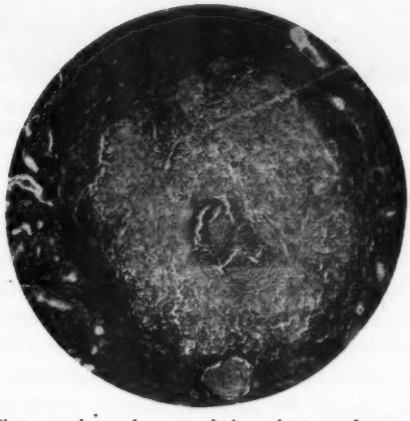

Fig. 6. The second conglomerate lesion, the central area of which shows a caseation. A proliferation of connective tissue is seen at the central area.

The third conglomerate lesion was situated in the cortex of the same lobe having the second conglomerate lesion but apart from the latter. The fourth conglomerate lesion was found on the surface of an upper renal lobe, where both the calyx and papilla were intact. The central areas of these third anfd ourth lesions were occupied mainly by connective tissue fibers showing a hyaline degeneration (Fig. 7), being surrounded by tuberculous granulation tissue and lymphocytes.

As mentioned above, the proliferation of connective tissue fibers was invisible in the smallest giant cell tuberlces but remarkable in the conglomerate lesions. In view of these facts, an examination of the degree of proliferation was made upon these fibers in the conglomerate lesions on the previously mentioned renal lobe having the main lesion, with the results that in the main lesion the proliferation was of an extent similar to that of the first conglomerate lesion, no scarring such as seen in the second to fourth lesions being observed.

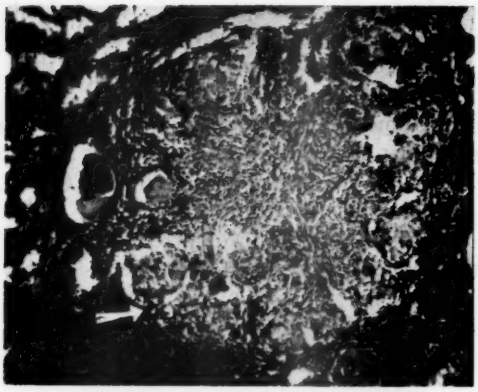

Fig. 7. The third conglomerate lesion, the central area of which is occupied by hyaline connective tissue fibers. The arrow indicates giant cells and epithelioid cells.

Results of examinations in Case 2.

The caseous lesion in the upper part of the kidney of Case 2 occupied two fused papillae and extended as far as the corticomedullary zone, and a large number of tubercles were found in the renal lobes connected with these papillae. Almost all these tubercles seem to have been ensued in the caseous papillary lesion as in Case 1. Even on a single section there were found a few tuberculous nodules in the corticomedullary zone, and several such nodules around the arciform artery, which had been produced pyelonephritically by the lesion at the calyx. Among these nodules an extinct scarred lesion was found, being surrounded by hyaline connective tissue fibers (Fig. 8). Thus, this scarred lesion also may be taken to be the oldest one that had ensued from the papillary lesion, though the possibility that this had been formed hematogenously prior to the development of the papillary lesion can not be excluded.

Though the mucosa of the lower major calyces were intact to the naked eye, microscopically a subepithelial tubercle formation was perceived on rare occasions; the papillae were intact also microscopically, but serial sections revealed six of the smallest tubercles in the cortex.

All these six tubercles were lesions mainly consisting of epithelioid cells surrounded by a layer of lymphocytes; due to a large extent of the lymphocyte infiltration the tubercles were fairly larger than the smallest giant cell tubercles in Case 1. Giant cells were found in two of these tubercles. As for the site of formation, one was formed in the corticomedullary zone, one on the renal surface, and four in the middle part of the cortex; two of the six were found close to and four apart from the

Fig. 8. A scarred lesion in the renal lobe having the main lesion (Case 2). A remarkable round cell infiltration is seen around the arciform artery.

glomerulus, being situated interstitially in all cases.

In the middle part of the kidney, that is, in the part intervening between the upper diseased and the lower relatively normal part, four smallest tubercles (3 epithelioid cell tubercles and 1 giant cell tubercle) of equal quality were found, one close to and three apart from the glomerulus, in the cortex of the area where a profound tuberculous lesion affected the calyx mucosa but not the papillary region; the pyelonephritic course in the formation of these tubercles were not clear, however.

Thus, in the relatively normal area in Case 2 there were found a total of 10 smallest tubercles (7 epithelioid cell tubercles and 3 giant cell tubercles), three of which were situated close to and seven apart from the glomerulus.

Results of examination in Case 3.

In the lower part of the kidney there existed a large lesion with a cavitation, more advanced than in the remaining two cases. Tuberculous changes were so profound also in each lower minor calyx that a large number of tubercles were formed in the parenchyma of the lower part of the kidney. Also in this case, these tubercles seem to have supervened in the calicine and papillary lesions.

Serial sections of the upper relatively normal area revealed three smallest tubercles consisting mainly of epithelioid cells; no giant cells were detected. Two of these were situated on the renal surface, their surrounding layer of lymphocyte infiltration being wedge-shaped and enclosing the glomerulus therein. The remaining one tubercle was found in the cortex of the corticomedullary zone, close to the glomerulus; though the papilla in this area was found normal, a subepithelial tubercle forma-

tion was seen on the calyx mucosa.

Discussion

If Medlar's hypothesis can be applied to the development of renal tuberculosis, close examination of a large " normal " area of a surgically removed tuberculous kidney with a localized major lesion may reveal in the cortex those tubercles hematogenously produced prior to the establishment of the major lesion which healed to an extinct scarred lesion or which aggravated and broke into the renal tubule, thus showing the possibility of becoming the origin of a papilla-calyx lesion. With such an expectation close examination was made in the three cases in the present investigation. Tubercles to be under consideration here are, therefore, those tuberculous ones which were found in a relatively normal area of the kidney.

Upon close examination of a total of 31,322 serial sections prepared in Case 1, 58 solitary tuberculous lesions were detected in the relatively normal area. Most of them were minute tubercles of the order of magnitude of a glomerulus, 52 of which were giant cell tubercles, two being epithelioid cell tubercles. Examination of more than 5000 serial sections prepared from the relatively normal area in Case 2 revealed seven epithelioid cell tubercles and three giant cell tubercles, while three epithelioid cell tubercles were detected on more than 4000 serial sections of the relatively normal area in Case 3. Common to Cases 1, 2 and 3, these smallest tubercles were characterized by being almost devoid of connective tissue fibers, which fact seems to indicate that these tubercles were relatively fresh.

Different from these smallest tubercles, in the four conglomerate lesions seen in the cortex of Case 1, the outermost layer consisted of lymphocytes and the middle layer had tuberculous nodules with giant cells or tuberculous granulation, while the central area consisted of proliferated connective tissue fibers and epithelioid cells in the first conglomerate lesion, of a small caseous lesion surrounded by connective tissue in the second, and solely of hyaline connective tissue in the third and fourth.

Medlar's interpretation of the tuberculous process may be summarized as follows: An epithelioid or mononuclear tubercle is first formed (the first stage of a tuberculous lesion), then an abscess is formed there (the second stage), and finally caseation supervenes and giant cells appaer (the third or reparative stage). Following caseation the mononuclear leucocytes and lymphocytes are attracted in large numbers. As a reparative process goes on toward completion the need for mononuclear leucocytes and lymphocytes becomes less and less until the product is a scar.

According to this viewpoint, the results of examination of the smallest tubercles in the above three cases may be represented as follows: In Case 1, a large majority of the tubercles were giant cell tubercles in the

reparative stage, though a few of them were epithelioid cell tubercles in the second stage; in Cases 2 and 3 it may be taken that there were found a few tubercles in the second stage and a few tubercles in the third, reparative stage; in addition, there was observed a proliferation of connective tissue fibers at the central area of the conglomerate lesions as seen in Case 1, the extent of the proliferation being of the increasing order of the first, second, third, and fourth lesions; in particular, the central area of the third or and fourth lesion consisted solely of connective tissue fibers and might be called a scar; this scar, however, was surrounded by a tuberculous lesion in the reparative stage.

The conglomerate lesions found in the normal area in Case 1 may be taken as those which had advanced further from the repearativ sgage. Comparing this with the tubercles that had ensued in the cortex of the renal lobe having the main lesion, a proliferation of connective tissue to such an extent as that of the first conglomerate lesion in the normal area was seen, but such instances of scarred tubercles as occurred in the second to fourth conglomerate lesions were not found. If tissue reaction of the cortex is assumed to be of the same extent in various parts of a kidney, the second to fourth conglomerate lesions may be taken to be older than those lesions that had ensued in the cortex of the renal lobe having the main lesion.

The process of formation of the smallest tubercle in a normal area can be inferred in various ways. As it is strongly possible that this formation is either hematogenous in origin or due to pyelonephritic spreading, these two possibilities have to be examined here. According to Medlar, Band, and others, the earliest tuberculous lesion hematogenous in origin usually appears in relation to the glomerulus of the renal cortex or in the capillary between the collecting tubeuls in the pyramid (Medlar).

In the present investigation, the sites of tubercle formation of the smallest size in the cortex of the normal area in Cases 1 to 3 were as follows: Of the 62 tubercles, seven were found in the superficial part of the renal cortex, 23 in the corticomedullary zone, and 32 in the intermediate area; of the 65 smallest tubercles (including those found at the renal coloumns), 24 were situated close to the glomerulus, while the remaining 41 were in the interstice, apart from the glomerulus, there being found no tubercles to have been formed at the glomerulus itself. It has not been clear whether the smallest tubercles as found in the interstice of the cortex were hematogenous in origin.

It has been clearly shown by Stoerk,[9] Putschar,[10] and others that surgical tuberculosis will, when formed in the papilla-calyx area, extend pyelonephritically into the renal parenchyma, and this was confirmed by Muto, Maeda and Murai,[11][12] of our Clinic. This " pyelonephritic ex-

tension" seems to be synonymous with the "ascending reinfection of the kidney" as described by Lieberthal. According to the results obtained at our Clinic, in the renal parenchyma where the major lesion is restricted to a certain area but tuberculous pyelitis extends from this lesion, a lymphocyte infiltration will invade, though the papilla is intact, along the interlobar and arciform arteries from the calyx mucosa. Occasionally there are found tubercles at several places, which may be regarded as pyelonephritic extension.

As already mentioned, in many cases no changes were found on the papilla, minor calyx and mucosa of the lobe where tubercles had been formed. In some cases, however, a small tuberculous lesion was observed at the minor calyx niche. It has not been proved clearly that these smallest tubercles has not been proved clearly that these smallest tubercles had been formed pyelonephritically.

Thus, from the findings of the present investigation, no definite conclusion can be drawn as to whether the tubercle formation was pyelonephritic or hematogenous. However, if assumed to be hematogenous in origin, these tubercles are taken to have been formed later than the main lesion. Hence, these tubercles were possibly produced at the time of hematogenous outburst of tubercle bacilli after the main lesion had been formed. In the central areas of the second, third and fourth conglomerate lesions in Case 1 there were observed tuberculous lesions in a stage older than that of the tubercles found in the renal lobe having the main lesion. These tuberculous lesions may be regarded as the scarred tubercles formed hematogenously before the main lesion was produced, although the tubercles were not completely scarified but surrounded by tuberculous lesions in my cases.

Further, among the tuberculous lesions in the normal area, no lesion was found to have aggravated and broken into the tubules, such as found by Konno and Abe[13] in the kidneys obtained upon authopsy of patients with pulmonary tuberculosis. This difference may be due to a high vividness of the surgical case as compared with the exhausted state of the cases with severe phthisis.

The hypothesis on the development of renal tuberculosis as put forward by Medlar, Lieberthal, Band and others is quite a clear-cut one. It seems difficult, however, to find its base in surgically removed kidneys.

Summary

Three specimens of surgically removed tuberculous kidney with localized major lesions and with large "normal" areas of the renal parenchyma were examined by the serial section method, with the following results:

1. In the normal renal parenchyma, that is, all the parts except the renal lobe having the main lesion, there were found 58 isolated tubercles in Case 1, 10 in Case 2, and three in Case 3. Many of these tubercles were epithelioid cell tubercles or giant cell tubercles, in no cases was a proliferation of connective tissue fibers observed.

2. A proliferation of connective tissue fibers or scarring was seen at the central areas of the third and fourth conglomerate lesions in Case 1, but tuberculous granulation was detected in their surroundings.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

On the Minimal Effective Dose of Noradrenaline for Causing the Blood Pressure Elevation in Non-Anesthetized Dogs

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(Received for publication, September 14, 1953)

No data are available regarding the minimal effective dose of noradrenaline for the blood pressure elevation in animals under physiological conditions. In order to supply such information, the present investigation was undertaken.

In addition, the pressor effects of L-noradrenaline and DL-noradrenaline were compared with that of L-adrenaline in non-anesthetized dogs.

Experimental

Methods and Results

(I)

Minimal effective dose of noradrenaline was determined in 12 healthy normal dogs weighing 5.0 to 13.8 kg.

The femoral artery was laid bare under local anesthesia with 1% procaine solution and arranged for the blood pressure recording. A small cannula for injection was inserted in the saphenous vein. The blood pressure tracings were taken by means of a mercury manometer. All the experiments were performed without anesthesia.

Synthetic DL-noradrenaline (Sankyo Co.) was dissolved with 1/10 n HCl as a 1 : 1,000 dilution and just before the injection further diluted with physiological saline solution. Noradrenaline solution was injected intravenously at a uniform rate of 1 cc. per minute for 5 minutes. The amount of noradrenaline injected in the present investigation, was 0.1 μg., 0.2 μg., 0.3 μg., 0.4 μg., 0.5 μg., 0.6 μg., 0.7 μg. and 1.0 μg. per kg. of body weight, per minute.

All the data are listed in Table I, and as an example the blood pressure tracings of Exp. 4 are illustrated in Fig. 1.

0.1 μg. of noradrenaline per kg. per minute was injected in 3 dogs.

TABLE I

Infusion of Dilute DL-Noradrenaline Solution to the Non-Anesthetized Dogs

No. of Exp.	Body weight and sex	Dose of noradrenaline injected per kg. per min. (μg.)	Blood pressure (mm. Hg.)		
			Before injection	During injection	Elevation
1	6.8 kg. ♂	0.1	123	119	−4
		0.2	114	117	3
		0.3	113	117	4
2	6.9 kg. ♂	0.1	89	86	−3
		0.2	95	94	−1
		0.3	88	90	2
3	8.2 kg. ♀	0.1	104	104	0
		0.2	105	109	4
		0.3	109	113	4
4	10.7 kg. ♂	0.3	126	132	6
		0.4	117	132	15
		0.5	121	142	21
5	10.2 kg. ♀	0.4	145	153	8
		0.5	138	156	18
		1.0	115	148	33
6	5.0 kg. ♀	0.4	165	170	5
		0.5	141	150	9
		0.6	142	153	11
7	7.0 kg. ♂	0.4	170	170	0
		0.7	157	168	11
8	8.5 kg. ♀	0.4	150	155	5
		0.5	116	132	16
		0.6	146	157	11
		0.7	148	161	13
		1.0	152	169	17
9	8.6 kg. ♀	0.3	130	133	3
		0.4	145	150	5
		0.5	116	129	13
		0.6	131	152	21
10	10.1 kg. ♀	0.5	128	132	4
		0.6	143	151	8
		0.7	142	155	13
11	7.7 kg. ♀	0.4	137	142	5
		0.5	151	161	10
		0.6	140	162	22
12	13.8 kg. ♀	0.2	159	162	3
		0.3	164	171	7
		0.4	158	168	10
		0.5	149	167	18
		0.6	156	172	16

In two cases the blood pressure descended slightly and in one case no change of the blood pressure was observed.

The injection of 0.2 μg. noradrenaline per kg. per minute was performed in 4 dogs. A very slight fall of the blood pressure was observed in

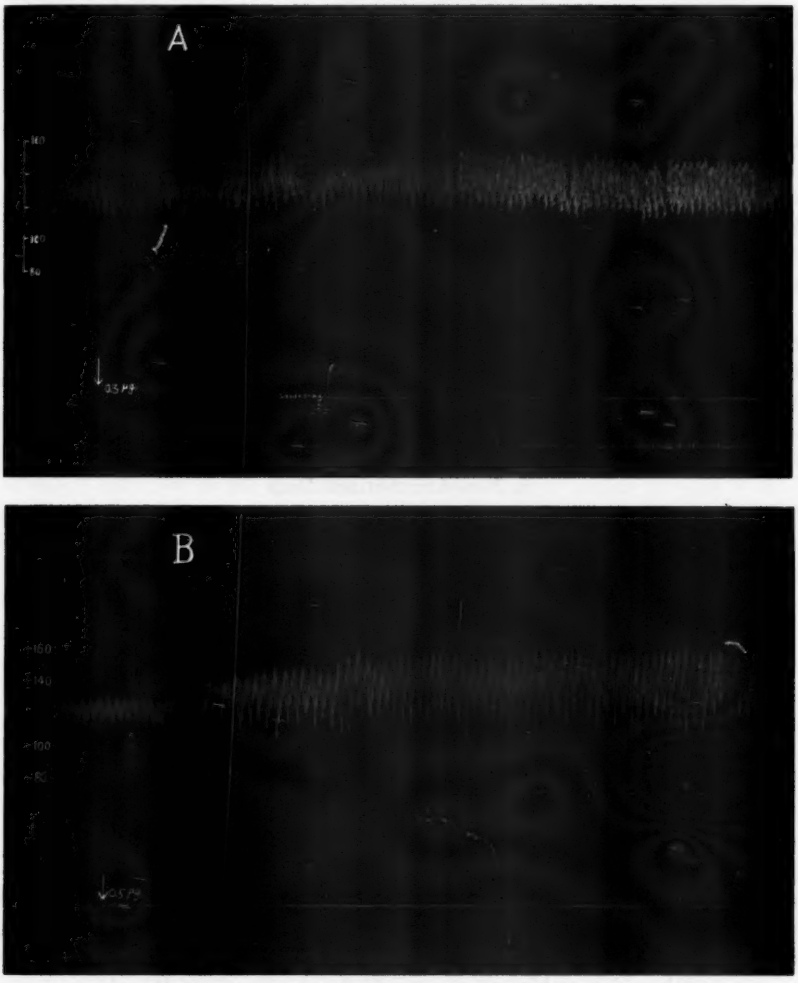

Fig. 1. Records of the blood pressure in Exp. 4. (Reduced to 2/5).
Dog, 10.7 kg. ♂. Non-anesthetized, non-fastened. A: Injection of DL-noradrenaline in a dose of 0.3 μg. per kg. per min. B: The same in a dose of 0.5 μg. per kg. per min. Time in 6 seconds.

one dog, while in other 3 dogs it was raised by 3, 3 and 4 mm. Hg., respectively.

Noradrenaline in a dose of 0.3 μg. per kg. per minute was injected in 6 dogs, resulting in elevating the blood pressure by 2–7 mm. Hg.

The injection of noradrenaline in a dose of 0.4 μg. per kg. per minute was performed in 8 dogs. In nearly all the cases the pressor effect was observed. The blood pressure was raised by 5–15 mm. Hg. Only in one dog (Exp. 7) the blood pressure remained unaltered.

The injection of 0.5 μg. noradrenaline per kg. per minute was attempted in 8 dogs. The blood pressure elevated by 4–21 mm. Hg.

0.6 μg. (in 6 dogs), 0.7 μg. (in 3 dogs) and 1.0 μg. (in 2 dogs) noradrenaline were also tested. In all the cases a more marked rise of the blood pressure was observed.

In order to see the results of all experiments at a glance, the data are summarized in Table II.

The minimal effective dose of DL-noradrenaline for causing the elevation of the blood pressure was thus estimated in the present investigation to be 0.3–0.4 μg. per kg. of body weight per minute.

TABLE II

Blood Pressure Response to Each Dose of DL-Noradrenaline in Non-Anesthetized Dogs

Dose of noradrenaline (μg. per kg. per min.)	Blood pressure elevation (mm. Hg.)							
0.1	−4,	−3,	0.					
0.2	−1,	3,	3,	4.				
0.3	2,	3,	4	4,	6,	7.		
0.4	0,	5,	5,	5,	5,	8,	10,	15.
0.5	4,	9,	10,	13,	16,	18,	18,	21.
0.6	8,	11,	11,	16,	21,	22.		
0.7	11,	13,	13.					
1.0	17,	33.						

(II)

In 3 healthy normal dogs, weighing 4.6–12.0 kg., the effects of L- and DL-noradrenaline were compared to that of L-adrenaline on the blood pressure. The procedure of experiments was almost the same as that above mentioned.

L-noradrenaline-D-bitartrate-monohydrate (Sterling Winthrop), DL-noradrenaline (Sankyo) and L-adrenaline (Sankyo) were used. The amount of L-noradrenaline in 10 mg. of L-noradrenaline-D-bitartrate-monohydrate was taken as 5 mg. One cc. of the solution, containing nor-

adrenaline or adrenaline in a dose of 2.5–5.0 μg. per kg., was injected intravenously in 10 seconds. Marked elevation of the blood pressure was always observed.

In Exp. 13, the effect of 2.5 μg. L-noradrenaline was almost the same with that of 5.0 μg. DL-noradrenaline and these were far more stronger than 3.0 μg. L-adrenaline. 5.0 μg. DL-noradrenaline was somewhat stronger than 4.0 μg. L-adrenaline and almost as strong as 5.0 μg. L-adrenaline. Thus the activity ratios of L-noradrenaline : DL-noradrenaline : L-adrenaline were estimated in this dog to be 2 : 1 : 1.

In Exp. 14, the effects of 2.5 μg. L-noradrenaline and 5.0 μg. DL-noradrenaline were almost the same. These were weaker than that of 5.0 μg. L-adrenaline and a little weaker than that of 4.5 μg. L-adrenaline. 5.0 μg. DL-noradrenaline was just as strong as 4.0 μg. L-adrenaline. Activity ratios (L-noradrenaline : DL-noradrenaline : L-adrenaline) were determined in this dog to be 1.6 : 0.8 : 1.

Almost the same result was obtained in Exp. 15.

Discussion

The minimal effective dose of DL-noradrenaline for causing the blood pressure elevation was estimated in this investigation to be 0.3–0.4 μg. per kg. per minute. As L-noradrenaline was determined to be about twice as much effective as the racemic one, it may not be unfair to take it that the minimal effective dose of L-noradrenaline is 0.15–0.2 μg. per kg. per minute.

Some reports of the previous investigators (Dragstedt, Wightman and Huffman,[1] Wada and Kanowoka[2] and Ooisi[3]) have indicated that the minimal effective dose of L-adrenaline for raising the blood pressure in non-anesthetized dogs was about 0.2–0.3 μg. per kg. per minute. Comparing these values it may be concluded that the minimal effective dose of L-noradrenaline is somewhat smaller than that of L-adrenaline.

The pressor effect of L-noradrenaline in non-anesthetized dogs was found in the present investigation to be 1.6–2.0 times as much as that of L-adrenaline. This agrees well with the results of previous investigators tested in anesthetized dogs.

In dogs with morphine-ether anesthesia, Schultz[4] estimated the ratio of pressor activity of DL-noradrenaline to DL-adrenaline to be 1.5 : 1. Tainter, Tullar and Luduena[5] reported that in dogs under phenobarbital anesthesia, L-noradrenaline was 1.64±0.1 times as much effective as L-adrenaline. Luduena *et al.*[6] estimated in barbitarized dogs that L-noradrenaline was 1.70 times more active than L-adrenaline in elevating the blood pressure.

I have already studied, in conjunction with Drs. T. Suzuki and T.

Nakamura,[7] the activity ratio of L-noradrenaline to L-adrenaline on the blood pressure of non-anesthetized dogs, whose spinal cord was sectioned previously at the level of the lowest cervical segment. L-noradrenaline was found in that time to be 2.1 times more effective than L-adrenaline in the average value.

Summary

Injecting intravenously DL-noradrenaline at the uniform rate during 5 minutes, the minimal effective dose for causing the blood pressure elevation was determined in non-anesthetized normal dogs. It was estimated to be 0.3–0.4 μg. per kg. per minute.

In addition, the pressor effects of L-noradrenaline, DL-noradrenaline and L-adrenaline were compared in non-anesthetized normal dogs. L-noradrenaline was double as much effective as DL-noradrenaline and 1.6–2.0 times as much active as L-adrenaline.

As the pressor effect of L-noradrenaline is twice as much as that of DL-noradrenaline, the minimal effective does of L-noradrenaline for causing the elevation of the blood pressure in non-anesthetized normal dogs will be 0.15–0.2 μg. per kg. per minute.

My thanks are due to Prof. T. Suzuki for his helpful advice.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

On the Activities of the Medullary Extract of the Bovine Adrenal Gland Estimated by Some Biologic Tests, Using the Adrenaline-Noradrenaline Mixture as the Standard

By

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Comparisons of adrenaline estimates of the adrenal extract obtainable by some biologic and chemical tests were performed extensively by Kojima, Nemoto, Saito, Sato and Suzuki.[1] Generally speaking, the estimates obtainable by various methods were not identical with each other. This disharmony of adrenaline estimates would be, as was suggested by Kojima *et al.*, due to the presence of some substances allied to adrenaline in the adrenal medulla.

Since L-noradrenaline was recently isolated by Bergström, Euler and Hamberg[2] as the crystalline base from the adrenal gland of cattle, the presence of noradrenaline in the bovine adrenals together with adrenaline will be unquestionable.

However, it is still uncertain whether active principles other than adrenaline and noradrenaline are contained in the adrenal medulla in a significant amount or not.

Is the fact that the different adrenaline estimates are obtained from the same adrenal extract according to the method applied, as was observed by Kojima *et al.*, wholly due to the presence of noradrenaline along with adrenaline in the adrenal medulla?

To clarify this problem, we have undertaken in the present investigation to compare the activities of the medullary extract of the bovine adrenals in some biologic tests, such as the rabbit intestine segment method, the dog blood pressure and the cat paradoxical pupil reaction, with that of the adrenaline-noradrenaline mixture.

Experimental

Methods

The medullary tissue of the bovine adrenals was separated from the cortical tissue and was extracted with 10% trichloracetic acid. In the first place the extract was assayed chemically in terms of adrenaline and noradrenaline contents by the permanganate method of Suzuki and Ozaki[3]. Then the biologic activities of the extract on the rabbit intestine segment method, the dog blood pressure and the cat denervated iris were estimated, using the adrenaline-noradrenaline mixture as the standard. The adrenaline-noradrenaline mixture was prepared so as to contain just the same percentage of noradrenaline and adrenaline with the chemical estimates of the bovine adrenal extract. L-adrenaline (Sankyo) and L-noradrenaline-D-bitartrate-monohydrate (Sterling Winthrop) were used. The amount of L-noradrenaline in 10 mg. of the latter was taken as 5 mg. These were dissolved in 1/10 N hydrochloric acid solution and were stocked in a refrigerator. Just before the tests, these stock solutions were further diluted with physiological saline solution and the mixture was prepared.

It may be needless to say that, if no other active principles than adrenaline and noradrenaline are contained in the adrenal medulla in a significant amount, the activities of the extract estimated by different biologic methods using the adrenaline-noradrenaline mixture as the standard will be similar each other.

The procedure of the biologic tests was wholly the same as was used in our previous investigation.[4]

In the rabbit intestine segment method, the rhythmic movements of the intestine strip in the warmed diluted blood solution were recorded. Then the diluted blood solution containing a known quantity of the extract or the adrenaline-noradrenaline mixture was poured into the intestine chamber to replace the blood solution. Thus the activity of the adrenal extract on the movement of the isolated rabbit intestine segment was compared with that of the adrenaline-noradrenaline mixture.

In the test of the dog blood pressure, the transection of the spinal cord was performed at the lowest level of the cervical segments under ether anesthesia and two or three hours after the section the experiment was started without anesthesia. The diluted solution of the adrenal extract was injected intravenously.

In the tests of the cat denervated iris, the superior cervical ganglion was excised unilaterally under ether one week or more prior to the estimation. At the performance of the test, the animal was anesthetized with urethane. The diluted solution of the adrenaline-noradrenaline mixture or the adrenal extract was injected into the femoral vein.

Results and Discussion

With six specimens of the adrenal extracts, the estimations were performed. The results are summarized in Table I.

TABLE I

Comparison of Activities of the Medullary Extract of the Bovine Adrenals, Estimated by Some Biologic Methods Using the Adrenaline-Noradrenaline Mixture as the Standard

No. of extract	Chemical estimates of adrenaline and noradrenaline content				Biologic activities expressed in mg. equivalents of (adrenaline+noradrenaline) per g. of medullary tissue		
	mg. per g. of medullary tissue			per cent noradrenaline			
	adrenaline	noradrenaline	adrenaline + noradrenaline		rabbit intestine segment method	dog blood pressure	cat paradoxical pupil reaction
1	6.7	2.6	9.3	28	11.9	11.1	9.9
2	5.4	3.2	8.6	37	11.3	9.1	10.1
3	8.6	3.6	12.2	30	12.8	13.6	14.7
4	8.5	3.5	12.0	29	17.0	11.3	15.8
5	7.6	3.0	10.6	28	10.4	10.4	12.4
6	6.2	3.2	9.4	34	11.1	11.1	11.1
Mean	**7.2**	**3.2**	**10.4**	**31**	**12.5**	**11.2**	**12.4**

Using the permanganate method of Suzuki and Ozaki, the noradrenaline was found to be contained 28–37% of the total active principles. The sum of the adrenaline and noradrenaline content estimated with this chemical method was 8.6–12.2 mg. per g. of the medullary tissue and the average value was 10.4 mg.

Using the adrenaline-noradrenaline mixture as the standard, the sum of adrenaline and noradrenaline content was estimated by the rabbit intestine segment method, the dog blood pressure method and the cat paradoxical pupil reaction. The rabbit intestine segment method and the cat pupil method yielded almost similar value, i.e. 12.5 mg. and 12.4 mg., respectively. The dog blood pressure method gave somewhat smaller value, i.e. 11.2 mg. If we compare the estimates obtained by three methods case by case, it will be noticed that the differences of values are rather small in all cases and may be accounted for by errors inherent to the methods tested, except for the extract No. 4.

From the experimental results above presented, it is justifialbe to assume that the difference of the adrenaline estimates observed by Kojima *et al.* may be in the most of the cases due to the presence of noradrenaline together with adrenaline in the adrenal medullary extract.

Of the extract No. 4, peculiar values were obtained. In this case the

estimates obtainable by the rabbit intestine segment method and by the cat pupil method were 17.0 mg. and 15.8 mg., respectively, and were definitely larger than that given by the dog blood pressure method. The latter was 11.3 mg.

As the activity ratio of noradrenaline to adrenaline is the highest in the rabbit intestine segment method, the smallest in the cat paradoxical pupil reaction and the intermediate in the dog blood pressure,[4] the result obtained with the extract No. 4 can not be explained by the presence of simply two hormones in the adrenal medulla, i.e. adrenaline and noradrenaline.

In examining the experimental results of Kojima *et al.* concerning the adrenaline estimates of the bovine adrenal medullary tissue case by case, we were surprised to find that in Exps. No. 3 and No. 9 the ratios of the estimates obtainable by the three biologic methods were much the same as that in the extract No. 4 of the present investigation. In Exp. No. 3 of Kojima *et al.*, the adrenaline estimates obtained by the intestine segment method and the pupil method were 11.3 mg. and 11.2 mg., respectively, and that yielded by the blood pressure method was 7.4 mg. In Exp. No. 9, the estimate of adrenaline given by the intestine as well as by the pupil was 14.8 mg. and that yielded by the blood pressure was 9.6 mg. However, the standard solutions used in the experiments of Kojima *et al.* and of the present investigation are not the same. Adrenaline solution was used in the former and the adrenaline-noradrenaline mixture in the latter.

Summary

The present investigation was designed with the desire to know whether the active principles other than adrenaline and noradrenaline are presented in the adrenal medulla in a significant amount or not.

The medullary tissue of the bovine adrenal gland was extracted with trichloracetic acid. At first, the noradrenaline and adrenaline contents were estimated chemically by means of the permanganate method reported by Suzuki and Ozaki. The adrenaline-noradrenaline mixture was then prepared so as to contain just the same percentage of noradrenaline and adrenaline as the chemical estimates of the extract. Using this mixture as the standard the activity of the extract in three biologic tests was estimated quantitatively.

In five out of six specimens, the estimates obtainable by the three different methods were in every case almost similar each other. In one specimen, however, the estimate given by the blood pressure method was definitely smaller than those given by the intestine segment method and the pupil method.

Thus it can not yet be concluded that the active principles of the adrenal medulla are always soley two hormones, i.e. adrenaline and noradrenaline.

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The Tohoku Journal of Experimental Medicine, Vol. 60, No. 2, 1954

Experimental Studies on the Mechanism of the Auricular Flutter

By

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(Received for publication, September 17, 1953)

It is a well known fact that there are two hypotheses on the nature of auricular flutter, namely, ectopic theory[1)-5)] and the circus movement theory[6)-15)]. Lively discussion is still being conducted between the said two theories. The Journal, " Circulation " of April 1953, has taken up this disputing issue[16)].

Most noteworthy research results obtained recently, with respect to this problem, are the experiments by Rosenblueth[12)13)] and the analysis of Prinzmetal[5)]. When auricular muscle is electrically stimulated, fibrillation easily occurs but flutter is difficult to be induced, as experienced by any persons who tried to produce an auricular flutter experimentally. However, Rosenblueth has discovered that the flutter can be easily induced by electrical stimulation after blocking the muscular bridge between the orfices of superior and inferior venae cavae. This phenomenon is explained by Rosenbleuth as follows: " It suggests that the infrequency of occurrence is due to the difficulty of initiating one-way flutter waves around the dual obstacle. Even though a one-way impulse may meet one of the cavae end-on, and thus propagate unidirectionally around it, the presence of a bridge of conducting tissue between the two vessels will usually lead to two-way conduction around the second cava, with a cancellation of the two wave fronts." After the blocking, the cancellation does not occur, therefore the flutter is easily induced. Accordingly, Rosenblueth advocates that the phenomenon in his experiments support the circus movement theory.

On the other hand, research of Prinzmetal[5)] is an observation on the auricular flutter induced mainly by aconitine, demonstrated by a high speed color film; according to this observation, the contraction wave

started at the injected area spreading to whole auricular muscle. Of course, this supports the ectopic theory.

Which of these two opposite facts corresponds to an auricular flutter observed in the clinical cases is an interesting problem. Main object of our experiment has been directed to this point.

Experimental

Experiments to blook a Muscular Bridge between the Orfices of Superior and Inferior Venae Cavae

In the first place we attempted to ascertain whether the auricular flutter would occur easily after blocking the muscular bridge between the orifices of superior and inferior venae cavae as observed by Rosenblueth.

Animal used for experiments was dog weighing 5–10 kg., with the use of a narcosis by intravenous injection of pentothal or evipan. Chest has been opened, cutting pericardium, exposing heart. The muscular bridge has been compressed by 3–4 Pean's forceps, thus blocking the same. An electric stimulation has been employed by thyratron, 500–600 cycle per minute frequentcy of which has induced the auricular flutter. Flutter did not occur so easily by a frequency other than the said cycle. Frequency of flutter wave induced by this cycle was 450–550 cycle per minute, slightly less than the cycle at the stimulation.

By this stimulation in all cases where forceps we used, it was possible to induce an auricular flutter as shown in Table I. But in some cases where no forceps were employed, it was impossible. Moreover, although flutter occurred easily where forceps were used, while without forceps flutter occurred only when electric stimulation has been applied repeatedly. Flutter which occured without forceps usually stopped in a short period, but with forceps the flutter has sometimes continued as long as one hour. Above observation verifies the results of Rosenblueth. Furthermore, it was not recognized that flutter does not occur unless stimulation is applied to a special region. Flutter sometimes occurs even under stimulation of the appendage.

Table I

	Total number of experiments	Auricular flutter occurred	Auricular flutter did not occur
Without blockade	20	13	7
With blockade	24	24	0

Analysis of the Intra-auricular Conduction of the Excitation Wave

Since Lewis' investigation[6-9], analysis of intra-auricular conduction is

being made by comparing the intrinsic deflections, same as in analysis of the intra-ventricular conduction.

However researches conducted heretofore have been made mainly with respect to right auricle and no detailed observation has been made with respect to left auricle. This is due to the fact that left auricle is hidden behind the heart and very difficult to be explored. However, we thought that one of the reasons for not clearly defining the nature of auricular flutter is because the propagation of excitation in left auricle is not well observed.

So, electrodes of solder balls made of silver have been chained together by one insulated tape (Fig. 1), and the surface of left auricle has been explored by inserting this tape between both venae cavae and pulmonary veins. Leads from right auricle were made by needle electrodes. One of these leads or an intra-auricular cavity lead has been employed as a standard curve.

a) Sinus rhythm: The excitation process in the sinus rhythm analyzed by the method stated above is shown in Fig. 2 as a control.

Fig. 1

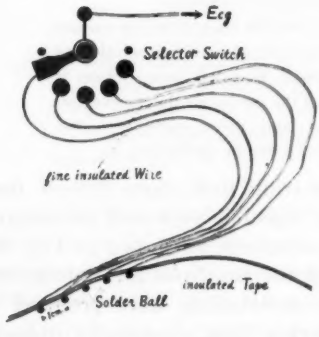


Fig. 2

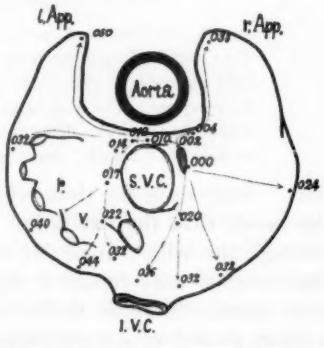


Fig. 1. A diagram of the electrodes used in direct leading from the left auricular surface.

Fig. 2. (No. 35, Exp. 1). Outline of the surface of a dog's auricle, showing readings for the arrival of the excitation wave during the sinus rhythm. The auricular cycle is 0.310 sec.

b) Aconitine flutter: Scherf[1)-4)] and Prinzmetal[5)] have studied the flutter which has been induced by injection of aconitine into auricular muscle as the object of their observation.

When 0.1–0.2 cc. of 0.05% water solution of aconitine is injected intramuscularly at any given region of an auricular muscle, a flutter-like condition occurs after a while. Fig. 3a shows the record of this condi-

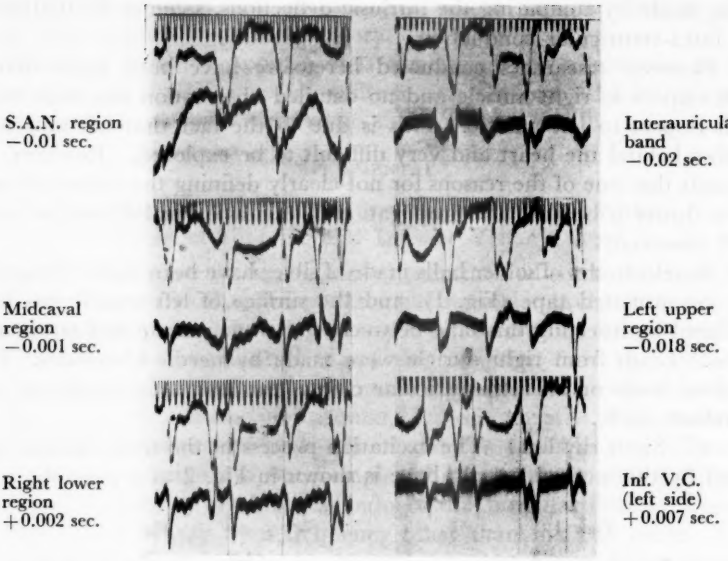


Fig. 3a (No. 35, Exp. 2). Curves taken by direct leads from the surface of an auricle during the flutter induced by injection of aconitine solution at right appendage. The curve taken simultaneously from left lower region is shown upper. The time figure shown at sides of the curves are the interval between the intrinsic deflections of the upper and lower curves. − and + indicate " before " and " after " the control intrinsic deflection.

tion. Analysisng the time relation of the intrinic deflections thereof, then the result that the excitation starts at an injected area and propagates through the whole auricular muscle, was obtained, as shown in Fig. 3b. Experiments were repeated eight times and the results of all experiments were same. Intrinsic deflections in these experiments were grouped in a short period of an auricular cycle, differing from electrically induced flutter which is explained below.

c) Auricular flutter induced by electric stimulation: Figures 4b and 5b are the analysis of propagation of excitation in auricular flutter induced by electric stimulation.

Analysing a time relation of its intrinsic deflections, they are distributed in order around the orfices of both venae cavae. Moreover, they are scattered all over one auricular cycle. This is judged to be a circus movement of excitation wave along the pathway as shown in Fig. 4b and 5b. Zero (0) indicated in the Figures does not mean that the excitation wave starts at this point; it merely means that the intrinsic deflection of this region is used as a standard of time analysis.

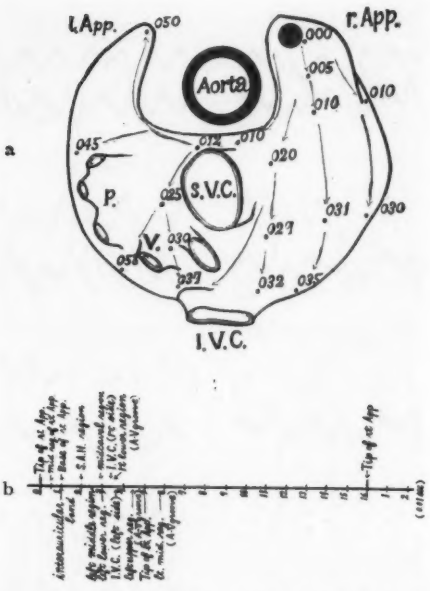


Fig. 3b (No. 35, Exp. 2). (a) Outline of the cephalic surface of a dog's auricle, showing the reading for the arrival of the excitation wave in the case of Fig. 3a. Aconitine solution was injected at the black shadowed portion. (b) A chart showing the time relation of readings from the surface of the auricle during the same flutter.

Twenty-six experiments were attempted and in each case the findings considered to be a circus motion has been obtained. Direction of circus motion is clockwise in some cases and counterclockwise in others, seeing from cranial side. Fig. 4 is a sample of a clockwise circus movement and Fig. 5 is that of a counterclockwise. No relation exists between the direction of circus movement and method of applying stimulation (seat of stimulated region, direction of the poles of stimulating electrodes, etc.). Of twenty-six experiments clockwise was eighteen and counterclockwise was eight. Also in the flutter induced without forcepts, it was possible to obtain a figure of circus movement (Fig. 6).

Further Observations on the Rosenblueth's Flutter

If the excitation is to move in circular way then the next problem will be where is the path of this movement. It is well known that the taenia terminalis was assumed by Lewis[6]. But according to Rosenblueth's experiments, even when the blocked area is widened beyond taenia ter-

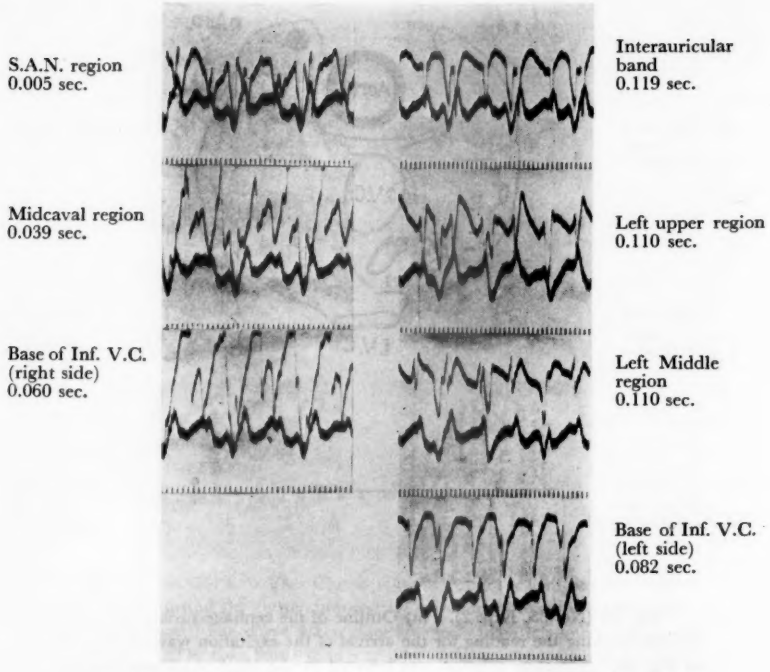
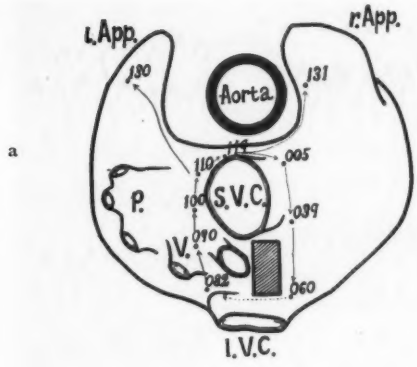


Fig. 4a (No. 12, Exp. 3). Curves taken by direct leads from the surface of a fluttering auricle, induced by electric stimulation at the caudal region of the right auricle. The curve shown lower was taken simultaniously from auricular cavity. The time shown at the sides of curves indicates the interval of the intrinsic deflection between those two curves. Auricular cycle is 0.130 sec.

minalis, flutter occurs. It stopped only when the injured area is enlarged to the auriculo-ventricular border.

The similar findings observed by us; the flutter does not stop by oppressing or cooling the stimulated place alone, while it stops when auricular body is oppressed or cooled from blocked area to auriculo-ventricular groove, as shown in Fig. 7. This fact indicates that circus way is not a limited path like taenia terminalis. Furthermore we observed the fact that, if an upper or lower part of the border between right and left auricle is cooled, the flutter stops easily. The reason therefore is thought to be due to the narrowing of muscle bundle in this region. From above facts we suppose that it is necessary for auricle muscle to be annular than the existance of a specific pathway in order that flutter occurs. Accordingly, if the length of this annular pathway is increased, frequency of flutter wave



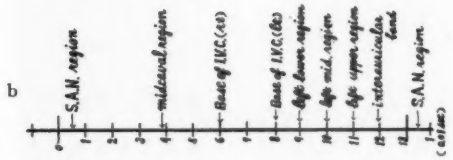


Fig. 4b (No. 12, Exp. 3). (a) Outline of the auricular surface constructed in the same fashion as Fig. 3b. The obliquely hatched area was injured by Pean's forceps. (b) A chart showing the time relation of readings from the surface of the auricle during the same flutter.

is expected to decrease. Rosenblueth recognized this fact. We have also observed a decrease in frequency when a blocked area is enlarged. But decrease of frequency of flutter was not so large as we have expected. Table II shows this fact. In this table the frequency of flutter wave computed from the enlarging rate of pathway is compared with the observed value.

The reason must be offered for such difference, but it seems to be difficult to give a definite answer. We suppose it as follows; although the excitation propagates through the auricular muscle annularly, it does not propagate perhaps around in a smooth ring. After all, it is necessary to consider an arrangement of each muscle bundle, therefore it is proper to assume that the excitation wave propagates along the ring in a zig-zag path. And if it is so assumed, frequency needs not necessarily to decrease so much as expected with an enlargement of the blocked area. This zig-zag propagation can be assumed from the fact that the intrinsic deflections are not distributed at regular intervals.

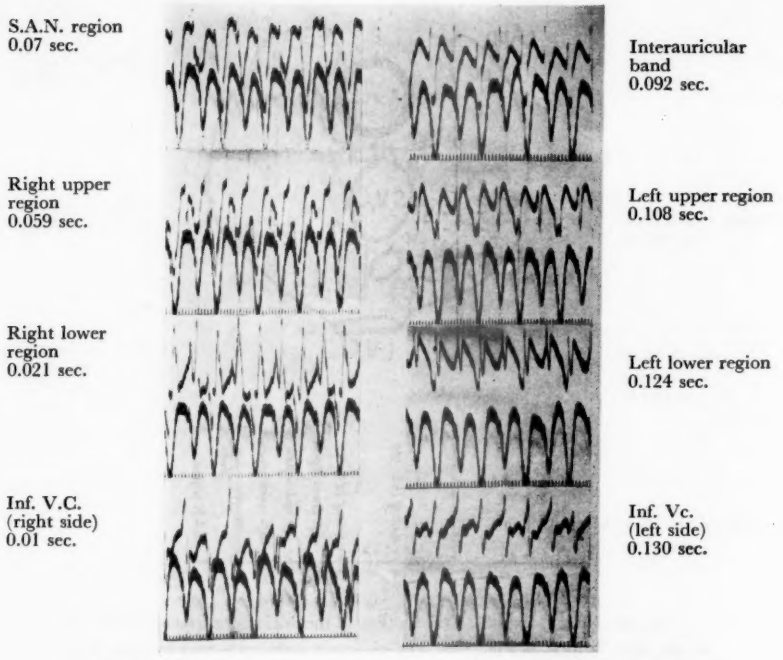


Fig. 5a (No. 12, Exp. 2). Curves taken by direct leads from the surface of an auricle during the flutter induced by electric stimulation at the caudal region of the right auricle. The lower curves were taken simultaneously from right auricular cavity. The time figures shown at the sides of curves indicate the intervals of the intrinsic deflections of the former from the bottom of the auricular complexes of the latter. The auricular cycle is 0.130 sec.

Dissimilarity between Aconitine Flutter and Electrically Induced Flutter

From the results of above mentioned experiments it is assumed that aconitine flutter and electrically induced flutter are different in nature. If so, then a problem arises as to the category to which a clinically observed flutter belongs.[10)15)] If those two flutters are comparatively observed, difference can be noticed at the time of returning to sinus rhythm. Electrically induced flutter returns to sinus rhythm instantly, while in the aconitine flutter auricular rate gradually decreases, then changes to sinus rhythm. This difference can be observed when a flutter is eliminated by using procaine amide. Because it is an established fact for a clinical flutter to convert to sinus rhythm instantly, so in the light thereof it can be assumed that a clinical flutter is under the same condition as electrically

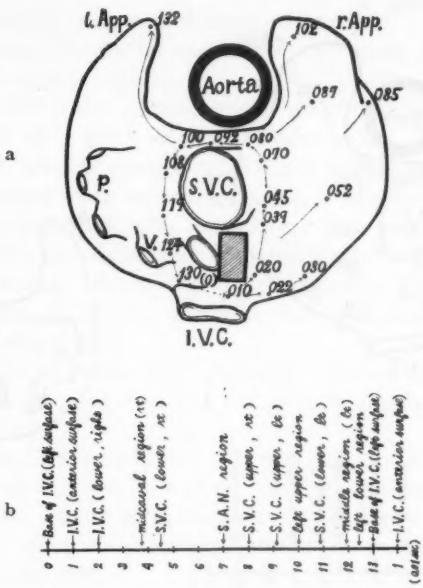


Fig. 5b (No. 12, Exp. 2). (a) Outline of the cephalic surface of a dog's auricle showing the arrival of the excitation wave, which have been obtained during the flutter same as Fig. 5a. The area plotted by oblique lines is injured. Auricular cycle is 0.130 sec. (b) A chart showing the time relation of readings from the surface of the auricle during the period of the same flutter.

TABLE II

Length of the Pathway has been Estimated from the Width of Injured Area

Experiment No.	Before widening of the blocked area			After widening of the blocked area		
	cycle length sec.	frequency per min.	length of pathway (estimated) cm.	length of pathway (estimated) cm.	calculated frequency per min.	observed frequency per min
1	0.130	461	12.0	14.9	370	414
2	0.120	500	12.0	13.0	461	480
3	0.125	480	14.0	15.5	401	428
4	0.130	461	13.0	15.0	393	414
5	0.115	522	12.0	14.0	447	454
6	0.122	492	14.0	16.0	430	423
7	0.110	545	12.0	15.0	467	545

Fig. 6

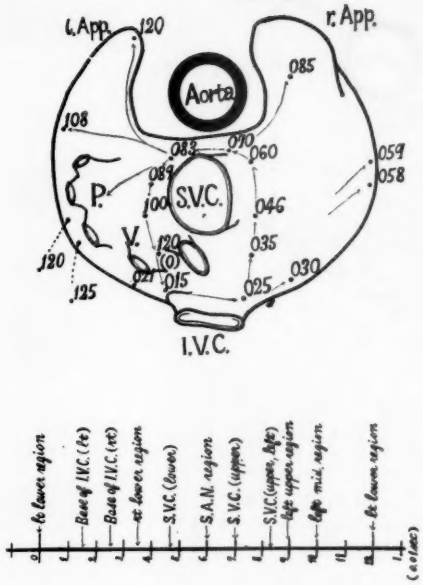


Fig. 7

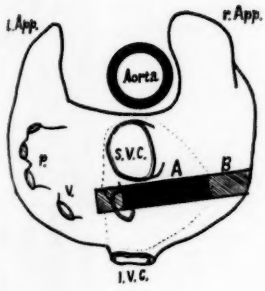


Fig. 6 (No. 29, Exp. 3). (a) Outline of the cephalic surface of a dog's auricle, showing the readings for the arrival of the excitation wave, which have been obtained during the flutter induced by stimulating at left appendage, without forceps. Auricular cycle is 0.120 sec. (b) A chart constructed in the same fashion as in Fig. 3b.

Fig. 7. A diagram showing enlargement of the blocked area. The flutter persists by the blocking of the area (A) alone, while it returns instantly to sinus rhythm when the blocked area is widened to the area plotted by oblique lines (B).

induced flutter, that is, flutter which occurs by circus movement of the excitation wave. This opinion was advanced by Brown and Acheson in 1952[14] upon the observation by the use of procaine amide. We agree with them.

Conclusion and Summary

1. As advocated by Rosenblueth auricular flutter easily and certainly occurs and continues for a long period, when a muscular bridge between the orfices of superior and inferior venae cavae is blocked.

2. 500–600 cycle per minute electric stimulation of auricular surface easily produces a flutter.

3. In inducing auricular flutter by electric stimulation it is not

necessary to stimulate a certain designated region.

4. In the aconitine flutter excitation wave starts at an injected area and spreads over whole auricle.

5. In the electrically induced flutter excitation wave surrounds and circulates around the orfices of both venae cavae. Direction of circus movement has no specific relation to method of stimulation.

6. Flutter stops when auricular muscle is oppressed or cooled so as to reach the auriculo-ventricular border from the blocked area. It does not stop when only a stimulated area or a neighbourhood of taenia terminalis is oppressed or cooled. Accordingly, circus way is not limited to taenia terminalis. Rather, it can be assumed that auricular muscle must be annular for inducing flutter.

7. Frequency of flutter wave decreases when a blocked area is enlarged, but not so much as expected. This indicates that the circus pathway is zig-zag ring.

8. Therefore, we concluded that electrically induced flutter and aconitine flutter are different in nature. And due to the fact that elimination of flutter in the former is instant while in the latter gradual, it can be assumed that an electrically induced flutter corresponds to the flutter observed in a clinical case.

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